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Development of an Automated Detection System for Nitrite in Aquatic Environments

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DEVELOPMENT OF AN AUTOMATED DETECTION SYSTEM FOR NITRITE IN AQUATIC ENVIRONMENTS

by

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ABSTRACT
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The main objective of the project is to develop an automated nitrite sensor for use in aquatic environments, and more specifically for use in recirculating aquaculture systems (RAS), where monitoring can help sustain a controlled environment, protect against nitrite intoxication, and promote fish health. Detecting nitrite manually with semi-quantitative colorimetric test kits, although inexpensive and simple, is prone to inter-user variability and poor sensitivity. An automated nitrite sensor has potential to provide higher resolution measurements at both concentration and time scales and can serve as a research tool for the study of filtration systems essential in maintaining a healthy RAS environment.

The questions driving the project are: How to build a device that can deliver satisfactory analytical merit (e.g., sensitivity, accuracy, precision), while maintaining reliable, inexpensive, and simple operation. The research involves investigation into detection methods and state of the art instrumentation available for nitrite, production trends in chemical total analysis systems, and centers around larger questions surrounding invention and innovation. The first steps towards such a device are benchtop prototyping of the detection and fluidic modules, their integration with wet chemistry, and the validation of the analytical process carried out by the

system. The project approaches the objectives with a design that relies on commercially available components and consumables and is modular and adaptable for future possible configurations.

To this end, the benchtop prototype was developed as an opto-fluidic system for automated colorimetric detection. With the exception of two custom-built PVC adaptors, the entire system was built with off-the-shelf parts for around $1,000. In addition to utilizing easily replaceable components, the system was tested using commercially available and pre-made reagents based on proven chemistry (Griess assay for nitrite). Preliminary results suggest the analytical process is capable of detecting sub-micromolar nitrite concentrations (limit of detection equal to 0.18 µM) at appreciable precision, sensitivity, and accuracy in comparison to commercial instruments.

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1. Chapter I

A path to impact for autonomous field deployable chemical sensors: A case study of *in situ* nitrite sensors

Abstract

Natural freshwater systems have been severely affected and altered by excess loading of macronutrients (e.g., nitrogen and phosphorous) from fertilizers, fossil fuels, and human and livestock waste. Impacts to drinking water quality, biogeochemical cycles, and aquatic ecosystems are estimated to incur costs of US$210 billion annually. Automated sensing technologies offer potential to support research and resource management efforts by providing sample in/answer out measurement services and acquiring higher resolution data than currently supported by conventional sampling methods at a fraction of the cost.

While research and development activities surrounding this technology have been ongoing for nearly four decades, automated field-deployable nutrient sensors (FDS) have not been widely implemented, practically adopted, or made accessible for the majority of users. This paper reviews the trends, opportunities, and challenges in production and implementation of FDS from a perspective of innovation and impact. We use nitrite sensors as a case study to characterize the user community and consumer needs, perform a content analysis on related publications, tabulate state-of-the-art examples and specifications, and discuss data life cycle considerations. With further development of FDS through prototyping and testing in real-world applications, these tools can deliver information for protecting and restoring natural waters, enhancing process control for industrial operations and water treatment, and novel research insights.

1.1. Introduction

Intensifying anthropogenic activities are necessitating an increased environmental monitoring effort to obviate water resource-related crises. While analytical chemistry techniques and technologies have rapidly improved, the cost and logistics of collecting and analyzing water samples remains prohibitory to adequately capture the real-time distribution of contaminants and nutrients in natural, drinking, and crop-sustaining waters across meaningful spatial scales (Sequeira et al., 2002; Prien, 2007b). To address this dilemma, a 'Grand Challenge' was posed to the environmental analytical chemistry community to develop capabilities to sample and monitoring air, water, and soil at higher frequency (Murray, 2010). To meet this goal requires approaches that greatly lower per-sample and per-measurement costs while improving methodologies and techniques for remote measurements (Murray, 2010).

Chemical-sensing field deployable sensors (FDS) are systems that operate autonomously in situ and offer the potential to solve the Grand Challenge (Cleary et al., 2013). A report for the United States' National Oceanic and Atmospheric Administration (NOAA) surveyed regulatory, academic, and industrial user groups recommended that emphasis should be placed on developing FDS for nutrients along with standardized criteria and nomenclature to assess their effectiveness (Koeppen et al., 1978b). Nearly forty years later, the idea of FDS and their integration into distributed sensing networks has increased in popularity and scope. FDS have been regarded as the 'the holy grail for environmental analysis' (De Marco et al., 2007) because of their potential to provide a solution to under-sampling problems in oceanographic research (Johnson et al., 2007) and revolutionize our understanding of environmental processes (Hart and Martinez, 2006), analogous to the comparison of movies with still photography (Prien, 2007a). The appeal for such instrumentation is reflected by a market potential of $150 million by 2020 in

the United States alone (ACT, 2015), however the technologies remain poorly implemented or adopted in environmental analytical chemistry (Rios et al., 2012; Cogan et al., 2013b). Current technological limitations and prohibitive costs hamper FDS inventions from becoming routinely and habitually used at a wide scale (ACT, 2014).

To become truly innovative, FDS must offer economic value and become widely adopted beyond their inventors and initial adopters (Garcia and Calantone, 2002). This process of transferring technology from research and development to commercialization and technological growth and maturity necessarily encounters uncertainties, risks, and consequences that must be overcome by developer, practitioner, and sponsor alike. In addition to reaching a Technology Readiness Level (TRL) (NASA, 2012; Mowlem et al., 2008) where uncertainty is low enough for commercial firms to invest in the technology and produce it on a mass scale, a user community willing to adopt and implement the technology must also be established. As developers improve technology and early adopters prove performance in real-world environments, market diffusion is more likely to spread (Moore, 2014). For FDS that reach high technology readiness, the development of functioning, practical, and low-cost FDS remains a fundamental yet extremely challenging goal necessary to cross the technological chasm (Nightingale et al., 2015; Radu et al., 2013). These concepts can be illustrated by overlaying elements of technology development such as the TRL scale and S-Curve of technological process with market-driven adoption behaviors (Figure 1).

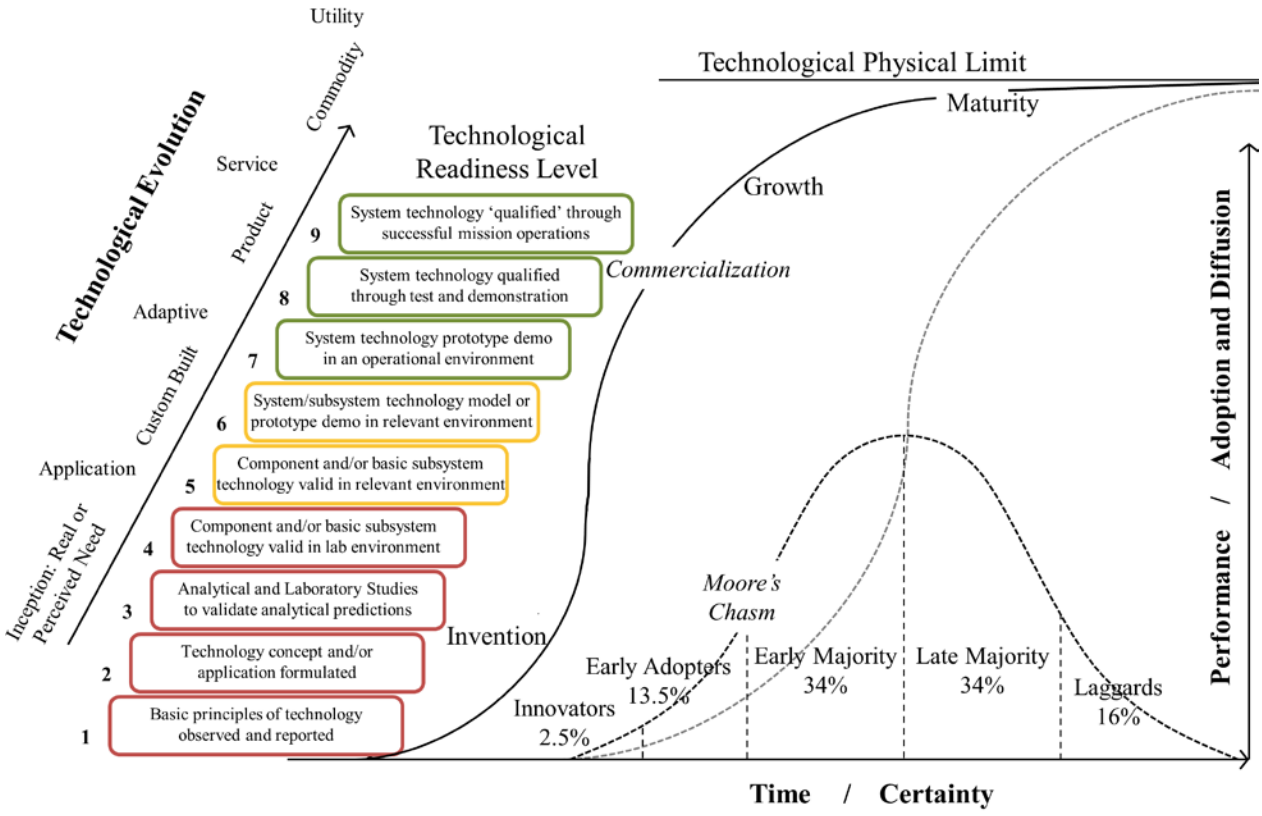

Figure 1. Technology readiness level and innovation. The process of invention (as given by TRL adapted from Mowlem et al., 2008) along the S-curve of Technological Process (solid line) coupled to the process of innovation (dashed lines), which comprises the entire cycle beginning with decision to undertake research followed by stages of development and commercialization along with market diffusion and finally the decision to adopt and implement (Rogers 1995). Together these processes iterate to advance a concept along a technological evolution scale (far left).

This review uses nitrite as a case study for the development and implementation of FDS in aquatic environments, highlights progress towards achieving answers for the Grand Challenge and addresses the requirements and challenges ahead. Nitrite has been selected as a parameter for evaluation for FDS because of its significance in aquatic environments and consequently its importance to environmental researchers and resource managers (Figure 2). Nitrite is also a chemical parameter that can be combined with nitrate and other macronutrients for analysis in FDS. We assess detection systems and state of the art instrumentation for nitrite as a proxy for

other spectrophotometric and electrochemical sensors, as the anion is subject to both forms of detection in situ.

1.1.1. Significance of nitrite in aquatic environments

As an intermediate compound in the overall nitrogen cycle, nitrite exists in aquatic environments at wide range of concentrations (Table 1), though most often at trace concentrations. Subject to abiotic and biotic transformation through photochemical degradation, nitrification, denitrification, and annamox processes, nitrite is an intermediate species that if accumulated can have significant negative impacts at global scales for human, animal, ecosystem, and economic health. Intensifying human activities (energy production, crop cultivation of legumes and rice, fertilizer and feed applications, human and animal waste, food preservatives) have more than doubled the amount of bioavailable nitrogen in the environment and increased the amount of nitrate and nitrite entering aquatic environments and water supplies (Wetzel, 2001). Though monitoring observations are routinely measured as total nitrate and nitrite (NO_x), there is an increasing desire to target nitrite specifically as we learn more about its role in physiological and environmental processes (Moorcroft et al., 2001).

Nitrite is considered more toxic than nitrate (Dutt and Davis, 2002), and can be responsible for methemoglobinemia, a condition that reduces the oxygen carrying capacity of red blood cells. This potentially fatal condition, along with nitrite's role as a suspected carcinogen (Moorcroft et al. 2001; Miró et al., 2003) and indicator of fecal pollution, have warranted legal recognition of nitrates and nitrites through thresholds in drinking water set by institutions such as the World Health Organization (WHO) and the United States Environmental Protection Agency (EPA) (Table 1). The risk for aquatic animals is even greater because of nitrite exposure at the

gill membrane (Kroupova et al., 2005), and necessitates continuous nitrite level regulation and monitoring in aquaculture operations (Table 1) especially in recirculating systems (Svobodova et al., 2005).

Table 1: Relevant ranges of nitrite in aquatic environments.

Matrix	Metric		Nitrite Concentration		Reference
Drinking Water	EPA National Standard		1 mg/L NO2-N (71.42 µM)		EPA, 2016
	WHO Standard		3 mg/L NO2 (65.20 µM)		WHO, 2011
	European Union Water Directive Standard		0.1 mg/L NO2 (2.17 µM)		EU, 2015
Natural Waters	Geographic Area		Approximate Range	Lab Resolution	Mowlem et al., 2008
	Surface Ocean		0.1 - 200 nM	0.1 nM	
	Deep Ocean		0.1 - 5 nM		
	Estuarine		0.5 - 1.5 µM	0.01 µM	
	Coastal		0.1 - 2 µM		
Aquaculture	Median 96 h LC50 (Adjusted for 20 ppm Chloride)	Coldwater Species	4.6 - 9.4 mg/L NO2-N (0.33 - 0.67 mM)		Lewis and Morris, 1986
		Warmwater Species	6.4 - 144 mg/L NO2-N (0.46 - 10.28 mM)		
		Other Families	9 < x <106 mg/L NO2-N (0.64 < x < 7.57 mM)		
	Preferred Range for Fish Culture		<1 mg/L NO2 (21.7 µM)		Buttner et al., 1993
			<0.1 mg/L NO2 (2.17 µM) in soft water		
European Union Water Directive: Minimum Instrument Performance Characteristics	Trueness (standard error)		10% of [NO2]		EU, 2002
	Precision (standard deviation)		10% of [NO2]		
	Limit of Detection (5x Standard Deviation of Blank)		10% of [NO2]		

Nitrite concentration is also used to investigate algal community composition, microbial relationships, and nutrient stoichiometry and dynamics in natural waters, where it can range from sub-nanomolar to several micro-molar levels (Table 1). Though it has received little monitoring attention relative to nitrate, accounting for nitrite uptake and release in aquatic ecosystems results in more accurate and unbiased models of primary productivity (Malerba et al., 2012). Collecting information on nitrite's distribution and concentration can help solve unanswered questions surrounding our ever-evolving understanding of nitrite accumulation, microbial activity and production pathways (Santoro et al., 2013; Arrigo, 2005). Consequently,

accumulating this knowledge with improved tools and applying it to models can help mitigate effects of nutrient loading and climate change on marine, estuarine, and freshwater populations (Ma et al., 2014).

1.1.2. Nitrite FDS: Stakeholders and user community

Based upon a survey of the marine research community in the 1970's, Koeppen et al. (1978b) reported to NOAA's Office of Ocean Engineering a review of the state-of-the-art of marine instrumentations and their deficiencies. Koeppen et al. organized data needs' characteristics into six major categories, with the rationale that the first four category needs would dictate the requirements in the last two (Figure 2). From an innovation perspective, categories one through four represent a market demand or 'market pull' for FDS, while five and six represent technology supply or technology push concepts. Ultimately the authors recommended an emphasis on the development of automated and reliable in situ instrumentation for nutrients (Koeppen et al., 1978a). Recent surveys conducted by the Alliance for Coastal Technologies (ACT, 2005; 2009) and the American University Center for Environmental Policy (2014) serve as updated assessments by examining user needs and priorities, currently available FDS technologies, and barriers to their development. Together these surveys provide an informative overview of the characteristics of nutrient FDS users, applications, and needs (Figure 2).

In Koeppen et al.'s survey, nitrates and nitrites ranked as the second and third highest-rated chemical parameters of interest (below only dissolved oxygen), and fifteenth/sixteenth out of ninety-seven other physical, chemical, biological, geological and meteorological parameters (Koeppen et al., 1978b). The ACT surveys also found nitrates and

nitrites together leading all other nutrient parameters in regards to measurement frequency and interest (Figure 2).

Koeppen et al. (1978a) favored government users of FDS because of the sector's power to formulate legislative mandates to which the industry and academic parties respond. The U.S. government, for instance, has budgeted over $3.5 billion spread among eleven agencies in 2016 for ocean-related information services (Sea Technology Buyer's Guide, 2016). Operational, forecasting, regulatory, and monitoring operations were favored by Koeppen et al. (1978b) because of more immediate data needs compared to baseline and research applications. Monitoring and research applications of in situ nutrient data were the primary (>70%) interests of the ACT survey population, which was comprised mainly of respondents from academic or government backgrounds, as well as environmental non-profit organizations, a sector still in its infancy in 1978. Less than 30% of ACT respondents planned on using data primarily for regulation, education, policy, or communication purposes (American University, 2014).

Affordable and smart FDS can provide means to create, monitor, and enforce nutrient load limits and regulations for pollution abatement and policies (ACT 2015; Moscetta et al., 2009). This is especially true at the interface of surface waters and the built environment, where complex ecosystems are particularly susceptible to anthropogenic, environmental, and hydrological influences. Coastal waters, estuaries, lentic, and lotic systems act as bioreactors and climate change sentinels, are responsible for nutrient transformations, food web support, and water supply. These geographic areas represent the majority of ACT respondents' field operation locations, which can be characterized by their relatively high level of turbidity, biological productivity, accessibility (reachable within a day or week), shallow depths, and average temperatures (Figure 2).

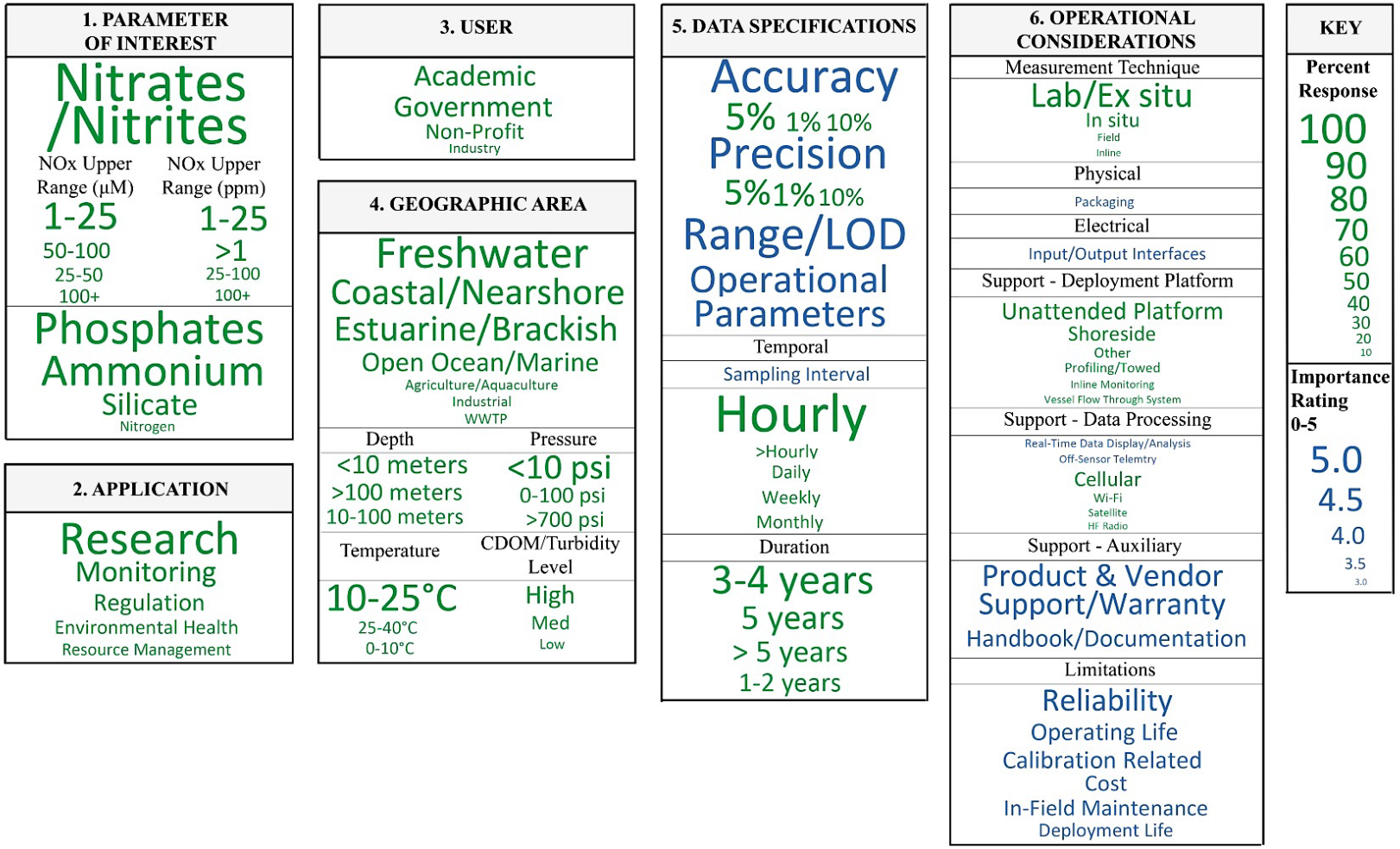

Figure 2. Tag cloud of nutrient FDS user needs, priorities, and characteristics. A qualitative visualization tool to provide a facile overview of recent ACT and American University survey information (2005, 2009, and 2014) overlaid within six major categories for marine environmental data needs (Koeppen et al. 1978a).

Over three-quarters of surveyed users collected nutrient data in the field and measured nutrients primarily ex situ, while nearly half of coastal professionals used in situ nutrient sensors some of the time, whether custom built packages (4%; 6%), commercial products (70%; 58%), or a combination of the two (26%; 38%) (ACT, 2005; 2009). The most common application for nutrient FDS was on a remote platform making continuous measurements, most often at an hourly interval. ACT respondents prioritized accuracy, precision, dynamic range, and operational parameters (sensitivity and resolution) in FDS for nutrient data over all other operational considerations (Figure 2).

1.1.3. Towards autonomous FDS: Technology outlook

Compared to inline cabinet analyzers, test kits, portable and handheld instrumentation, FDS offer the additional advantages of dynamic sampling strategies and remote autonomous operation that lowers costs associated with manual sampling efforts (e.g., personnel, travel, equipment, sample transport) (Moore et al., 2009). In addition, sampling and subsequent analysis on site also removes the risk of error and contamination associated with manual sample acquisition, storage, and transport. The portability and packaging of FDS enables deployment from a variety of platforms, including buoy systems, remotely operated vehicles and gliders, profilers, and underway systems (Adornato et al., 2010).

Operational considerations, while extraneous to pure 'data needs' are often the limiting factor for FDS and the area where instrument deficiencies are commonly expressed (Koeppen et al., 1978a). Moreover, the periphery subsystems of FDS ultimately limit reliability, accuracy, and durability in real-world environments. The design of the interface between the device and the environment is often underdeveloped (Marle and Greenway, 2005), yet altogether necessary

to make long-term autonomous deployments realistic (Campos and da Silva, 2013). In practice, FDS must detect their target analytes accurately and precisely (often at trace levels) while enduring hostile environmental factors, physical shock, self-correcting for instrument drift and stability, dealing with biofilm and particulate matter, and consuming minimal power. Inevitably these confounding environmental factors raise the cost of fabrication and design (Diamond et al., 2011). The ACT surveys found the most common constraints for FDS adoption were cost, lack of confidence in data and technical expertise. FDS limitations commonly cited were ease of calibration, overall reliability/durability, hardware/software data management, and range/detection limits (ACT, 2005; 2009). The overall complexity, technical demands, operating errors, and reliability are manifested in the form of state of the art FDS that cost $20,000 - $30,000, are limited to field deployments of a few weeks, and require an advanced level of training to operate (ACT 2015). These factors can result in a significant cost disparity between nutrient FDS implementation and traditional sampling and ex situ analysis. For example, a nitrate measurement performed by the US Geological Survey cost US$4,400 on average in 2013 (including salary, equipment and laboratory analysis), and the average cost of a discrete measurement by a FDS probe (including instrument acquisition, maintenance, and data validation costs) of $60,000 (Betanzo et al., 2015).

The uncertainties surrounding FDS could be mitigated with technical product support, a trait highly valued by the user community, and slightly less expensive systems ranging from $1,000 - $5,000 (ACT 2014). Realization of practical FDS involves satisfying both analytical requirements (minimal drift, resistance to biofouling, analyte specificity in complex matrices, and data validation of accuracy and precision) and technological requirements (production on a mass-scale, minimal power requirements, and robust electronics) (Radu et al., 2013; Zuliani and

Diamond, 2012). Maintenance and major service issues that result in unfeasible time and financial costs must also be addressed.

To make continuous monitoring with FDS realistic, plans of data handling, transmission, and quality assurance must also be in place. As potential solutions for these complex and interdependent concerns, emerging techniques for chemical detection and determination, instrument miniaturization, alternative energy, and wireless communication must prove their legitimacy in actual field applications and deployments. Sponsorship of the scientific merit behind these exercises associated with bringing inventions through TRLs 4-6 and beyond will be a key driver to their success (Prien, 2007a). To reach technological maturity and achieve an innovative reach, FDS will ultimately need to offer clear advantages over other sampling and measurement techniques; FDS must become cost-saving tools, practical in terms of trained personnel, interoperable with other sensor systems and platforms, and compatible with monitoring and research expectations and processes (Dragos et al., 2006). Until that point, we cannot expect FDS to become routinely depended on or adopted by the majority of users (Ho et al., 2005; Shade et al., 2009).

1.1.4. Detection Strategies

At its most basic definition, a FDS operates autonomously at the sampling location and turns a chemical quantity into an electrical signal, which is in turn processed and reported. The concept of a Total Analytical System (TAS) (Graber et al., 1990) applies to automation of the relevant phases of a quantitative chemical analysis, including sample introduction, sample transport, chemical reactions, chromatographic separations, detection, and transport to waste (Manz et al., 1990). Motivation for improved analytical performance and efficiency brought

forth the concept of the micro-Total Analytical System (μTAS) (Manz et al., 1990), a system capable of precisely handling volumes on the microliter level and performing assays in terms of seconds at or near the sampling site. Simultaneously, this miniaturization offered a convenient platform for portability and automation (Greenway et al., 1999). A realization and extension of a μTAS, a FDS is integrated with all necessary periphery subsystems working together to handle the mass flow and information flow of long-term, unmanned in situ chemical analysis.

The detection principle of any μTAS not only determines performance factors such as dynamic range, accuracy, and precision, but also influences downstream decision factors such as hardware, consumables, power requirements, maintenance, and limitations. Wide ranges of analytical techniques are available to quantify nitrite in aqueous samples, and consist of mature methods (i.e., spectroscopy, electrochemical) and relatively novel and emerging methods (i.e., biosensors). Optical approaches include reagent-based wet chemistry such as colorimetry based on the Griess assay (1879), fluorescence (Masserini and Fanning, 2000; Liu et al., 2009), chemiluminescence (Mikuška and Večeřa, 2003), and direct spectroscopy including ultra-violet absorption (Zhang et al., 2011; van den Broeke et al., 2006) and Raman scattering. Nitrite is a highly electroactive species and can be detected at metal, chemically modified, and enzymatic electrodes (Badea et al., 2001). Finally, biosensors may produce an optical or electrical signal, which is mediated by biological activity consisting of a bacterial community or specific enzyme(s) (Almeida et al., 2010).

Extensive reviews and examples of the performance and parameters of these detection principles for nitrite have been previously described (Yilong et al., 2015; Ma et al., 2014; Dutt and Davis, 2002; Moorcroft et al., 2001; Miró et al., 2003), and provide information on selectivity and sensitivity for nitrite across different matrices. Developers of FDS must first and

foremost consider the underlying chemistry of the method and the approach's stability across large temperature, pressure, and salinity gradients. Adjustments for chemical interferences from air bubbles, particulate matter, and complicated sample matrices must also be considered for long-term autonomous deployments (Campos and da Silva, 2013; Rios and Zougagh, 2013). Further challenges include the adaptation of the chosen method to meet storage demands, self-cleaning and calibration abilities.

1.1.5. Trends in the literature

1.1.5.1. Methodology

To investigate the trends in development and application of FDS for nitrite sensing, a content analysis was conducted on the body of literature describing the development of the major approaches for nitrite analysis and environmental monitoring. Searches through six databases (Google Scholar, SciFinder, IEEE Explorer, ProQuest Aquatic Sciences Collection, Compendex Engineering Database, and Web of Science Core Collection between the period of 2/3/16 and 3/7/16 were performed. Initial searches using keywords intended to be as broad as possible (e.g., 'Nitrite' and 'Sensor' and 'Water') were used to capture the most possible works. Relevant returns (based on queries of Title and Abstract fields) were collected and organized in EndNote (Web of Science). Removal of replicate entries produced a list of 1,102 records. Further processing separated literature into the main categories based on detection principles, and two categories based on Technology Readiness Levels (Mowlem et al., 2008). While this search process cannot capture every publication in this field, it will provide a comprehensive and representative sample as the databases queried cover the areas of chemistry, electronics,

engineering, and application. This is representative of the multi-disciplinary nature of FDS development and use.

The resulting database of works spanned over 40 years and contained over 350 different publication sources. Approximately 89% were published in academic journals, 6% in conference proceedings, and 5% in trade papers and patents. Of the academic journals, the top five journals containing the most publications were Analytica Chimica Acta (7.3%), Talanta (6.1%), Sensors and Actuators B (5.7%), Analyst (3.3%), and Electroanalysis and Electrochimica Acta (2.6% each). A full list of the search keywords and phrases for the overall methodology and is provided in Appendix A (Figure A.1).

1.1.5.2. Findings

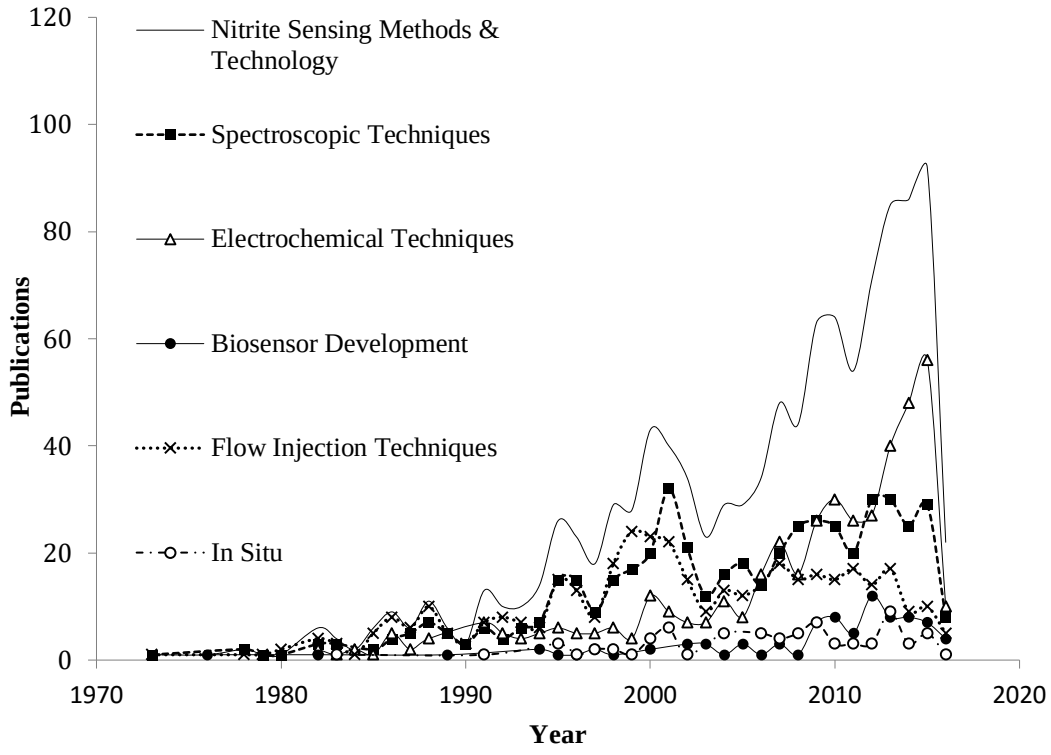

Figure 3. Analysis of peer reviewed publications for nitrite analysis. Publications regarding nitrite detection and determination in aquatic environments and associated category components over time.

Overall, the number of publications on nitrite sensing methods and technologies for environmental applications have tripled on average each decade (Figure 2). Since Murray's Grand Challenge, there have been over 1,100 reviews published on the topic of environmental analysis (Radu et al., 2013), and the number of publications pertaining to nitrite has risen to over 470 at an average of 75 per year (2010 – 2015, Figure 2). Each publication represents an investment of resources into development, review, and reporting of efforts to make nitrite detection more affordable, simplified, selective, sensitive, accurate, precise, and/or applied.

1.1.5.2.1. Trends in detection strategies

Among detection techniques, spectroscopic methods were found to be applied to sensors at a higher percentage (37%) than electro-chemical (28%) and biosensing (6%) approaches. Spectroscopic techniques consist mainly of the colorimetric method for nitrite, which remains the gold standard due to its economic and analytical advantages afforded by its relative simplicity, instrumentation availability, (Moorcroft et al., 2001; Dutt and Davis, 2002), high degree of accuracy and sensitivity (Gong et al., 2009; Hansen and Koroleff, 1999; Moscetta et al., 2009), as well as stability and linearity in a variety of environmental matrices (Ma et al., 2014; Sieben et al., 2010). Colorimetric methods can routinely achieve sub-nanomolar concentrations (Ma et al., 2014) as part of low-cost detection systems consisting of components such as LED light sources (O'Toole and Diamond, 2008; Bui and Hauser, 2015; Capitan-Vallvey and Palma, 2011), reverse-biased LEDs (O'Toole et al., 2007), transducers integrated into custom circuitry (Gong et al., 2009), and web cameras (Santos et al., 2016).

The relatively stable nature, proven sensitivity, and accuracy of spectrophotometric methods have been employed in instrument packages for field deployments and have resulted in

commercial realizations of FDS (Table 2). Early instruments were used as submersible profilers for shipboard use (Hanson, 2000; Masserini and Fanning, 2000; Thouron et al., 2003), and with autonomous floats, vehicles, and profilers (Steimle et al., 2002; Bryne et al., 1999; Adornato et al., 2005). The challenges for field applications of optical methods occur in turbid and complex sample matrices, where light scattering and refraction from colloidal and suspended particulate matter and chemical interferences require some form of sample pre-treatment or filtration. The need for reagents and the related concerns of stability and contamination can also be severe disadvantages for extending deployment lifetimes for sensors using wet chemistry (Dutt and Davis, 2002; Yilong et al., 2015; Bende-Michl and Hairsine, 2009). Finally, biofouling remains a significant limitation for long-term deployments (Adornato et al., 2010; Cleary et al., 2013; Campos and da Silva, 2013; Nightingale et al., 2015).

Electrochemical sensors are among the most widely used in situ chemical sensors for oceanographic research in general (Moore et al., 2009) and are anticipated to be the fastest growing sector of the chemical sensor market globally (Research and Markets, 2015). These sensors are highly valued for their measurement simplicity, immunity to colored and turbid waters, freedom from reagents, and low cost. Recently the desire to invest resources into developing electrochemical and biosensors for nitrite is evidenced by the rapid increase in their publication numbers over the past decade, when articles featuring electrochemical and biosensor design and development accounted for nearly 45% more publications than those implementing optical methods. For nitrite FDS, researchers have predicted this trend because of the attractive potential simplicity and low maintenance that comes without need of reagents (Dutt and Davis, 2002; Bende-Michl and Hairsine, 2009).

Though electrochemical techniques are mature, well documented, and have been in use since the early 1900s (Yilong et al., 2015), nearly 50% of the publications concerning electrochemical publications for nitrite came after Murray's Grand Challenge in 2010. Much of the literature in this area has been devoted to making improvements to sensitivity, selectivity, and stability of voltammetric electrodes through surface modification and experimentation using new substrates, nanomaterials, and electroplating techniques. As a result, voltammetry has proven to be very sensitive and selective in controlled environments. Malha et al. (2013) reported a 5 nM Limit of Detection (LOD) with 2.5% relative standard deviation for nitrite concentration using a carbon black on solid paste electrode in a laboratory environment. Still, application to field analysis and commercialization is extremely limited due to interferences in complex matrices and stability issues in real-world environments. Though the use of carbon nanotubes have extended electrode stability to well over a month's time in the lab (Zhou et al., 2013; Zhang et al., 2013), sensor response and performance is severely compromised when exposed in situ, where other oxides, gas bubbles, and ions adsorb to the electrode surface more frequently and rapidly (Yilong et al., 2015; Dutt and Davis, 2002). Dutt and Davis (2002) described the literature related to these methods as more curiosity-driven than applicability-driven; 15 years later, the aforementioned operational requirements still constrain voltammetric-based sensors for a long-term, autonomous FDS.

Potentiometric classes of electrochemical sensors, or ion-selective electrodes (ISEs), represent the other major class of electrochemical sensors. Solid state, liquid-based, or compound sensors detect species activity at the interface of the sensor membrane and sample, where establishment of chemical equilibrium leads to a change in voltage that is compared to a reference electrode. Because of the membrane's ion-specific affinity and transport ability, ISEs

are highly selective, low power, and do not consume or produce chemical species (Harris, 2007; Hanrahan et al., 2004). These benefits make ISEs robust, simple to fabricate, easy to use, low cost, and portable; as such, they have become the favored electrochemical approach for in situ analysis and commercialization (Zuliani and Diamond 2012; De Marco et al., 2007; Radu et al., 2013; Dutt and Davis, 2002). Additionally, ISEs are versatile: they can be combined to detect multiple species within the same instrumental setup, have a wide operating detection range (spanning up to 12 orders of magnitude), and offer the greatest potential for fast measurements (seconds to minutes) (Bende-Michl and Hairsine, 2009; Radu et al., 2013).

For all of their practical advantages, ISEs are generally implemented for screening and confirmatory monitoring rather than research investigations characterizing subtle changes in aquatic environments (Dutt and Davis, 2002; Radu et al., 2013; Yilong et al., 2015). Though ISEs do not offer the sensitivity and precision of voltammetric and wet-chemistry sensors, their analytical performance has improved thanks to theoretical-based developments over the turn of the last century (Zuliani and Diamond, 2012; Bakker et al., 2011; Radu et al., 2013). An example of such improvement is given by Prasad et al. (2004), who reported development of a polymer-membrane potentiometric nitrite sensor capable of reaching a 1.0 μM LOD that could be stored for 5 months in a 0.1 M NO_2^- solution.

With the combination of steadily improving analytical attributes and overall practicality, ISEs show great promise and potential for their application as FDS for nitrite. When it comes to in situ and automated operation, however, the challenges facing their deployment are not easily overcome as ISEs suffer from similar problems that plague optical and other electrochemical sensors: Interferences in complex matrices, biofouling, and instrument drift. For the potentiometric sensor, operational problems are manifested in corrupted and passivated

membranes caused by interferences from other anionic species, biofouling, and mechanical shock or physical damage from debris in the water. These issues can result in sluggish response, highly unreliable data and erratic behavior (De Marco et al., 2007; Harris, 2007; Yilong et al., 2015; Radu et al., 2013). These problems are compounded by the fact that polymeric membranes, the most versatile and sensitive, are quite fragile compared to crystalline membranes, have a limited shelf life and may leach components into the sample solution (Radu et al., 2013; De Marco et al., 2007, Harris, 2007).

1.1.5.2.2. Flow injection technologies

Flow injection analysis (FIA) as an umbrella term encompasses the application of automated fluidic technologies and techniques to an analytical chemical process. There has been an increasing awareness and application of FIA in the form of microfluidics, with over 1,700 publications from 2004 – 2013 (Antony et al., 2014). Varying forms of FIA including segmented, sequential, reverse, and micro-fluidic injection techniques have been employed most frequently with optical detection methods in literature regarding environmental measurement of nitrite (Figure 3). The use of FIA-related technologies with optical detection methods represents a higher degree of autonomous operation than other detection methods (e.g., electrochemical, biosensors) have achieved.

Reviews of applications of FIA techniques to analysis of dissolved nitrite, among other target analytes, showcase the actuators, pumps, fabrication, and technologies necessary to make FDS use possible from vessels and in situ deployment platforms (Miró et al., 2003; Worsfold et al., 2013; Nightingale et al., 2015). Flow propulsion and operational function with electro-osmotic flow, increasingly miniaturized pumps and actuators, bio-mimicking ionogels, multi-

commutation strategies, and lab-on-chip/lab-on-valve fabrication have been reported to save time and minimize cost, reagents, size, power, and waste (Greenway et al., 1999; Feres and Reis, 2005; Cerdá et al., 1998; Wu and Ruzicka, 2001; Sieben et al., 2010; Zhang et al., 2009; Melchert et al., 2007; Rodenas-Torrabla et al., 2006; Czugala et al., 2013a). The trends of FIA-related applications to nitrite sensors appear to correlate well with literature also utilizing spectroscopic methods (Figure 3), with 75% of publications appearing after 1995 and 50% after 2000. Despite the advances in microfluidic technologies and applications, the number of publications with FIA-related keywords experienced a slight decrease (2%) when comparing the last 10 years (2005-2015) to the previous decade (1995-2005). Because the components involved in fluidic handling often make up the bulk of the cost in terms of instrument complexity, power consumption, overall size, and financial expense of a chemical analyzer (Cogan et al., 2013), there may be a trend to move towards reagent-less electrochemical or spectroscopic sensors which require fewer moving parts and less overall complexity.

1.1.5.3. In situ examples and state of the art

Though there are examples in the reviewed literature describing the development of custom built FDS across TRLs 4-6 (Figure 1), (i.e., Nakatani et al., 2004; Park et al., 2004; Hirata et al., 2003), and underway monitoring applications, (Li et al., 2008; Chen et al., 2008; Chavez et al., 1991; Petersen et al., 2006; Petersen et al., 2014), only around 2% qualified as describing FDS TRL 7 or above. This lack of literature supports Mowlem et al.'s assertion (2008) and reveals there is still a need to fill the gap when it comes to reporting actual deployments and field trials.

In 1986 Johnson et al., described the SCANNER, an automated submersible flow injection system with spectrophotometric detection. Since that time, there have been several examples of nitrite FDS that have been commercially produced and successfully deployed and reported on by the research community. First generation submersible chemical analyzers at the turn of the century were used in cabled and profiling oceanographic research applications and were characterized by flow injection schemes coupled with spectrophotometric detection and multi-parameter analysis capabilities. Commercial and custom-built systems such as the Spectral Elemental Analysis System (SEAS) (Byrne et al., 1999; Byrne et al., 2001; Kaltenbacher et al., 2000, 2001), the AquaSensor (Dunning and Sawkins, 2000) and the SubChemPak Analyzer (Hanson, 2000) proved that the concept of bringing the laboratory to the field could be effective by measuring and recording nitrite concentration values at higher temporal and spatial resolutions than previously possible, allowing researchers to measure high resolution vertical gradients, map plumes, and identify primary nitrite maxima. On-board standards, cleaning agents, and dual-path cells enabled accurate measurements and background corrections for optical drift, absorbance, and scattering. The sensitivity of these wet chemistry analyzers rivaled laboratory analysis with reported detection limits down to 1.0 nanomolar (Hanson, 2000). However the high investment cost and maintenance requirements associated with instrument operation and upkeep of components such as system consumables, fiber optics, lamps, diffraction gratings, liquid core waveguides, valves, and pumps along with reliability issues (fluidic errors, breakages, optical interferences) (Worsfold et al., 2013; Fay et al., 2011) severely limited the practical use and widespread adoption of these systems.

The current generation of wet chemistry FDS (Table 2) have taken advantage of advances in hydraulic and electronic technologies to produce smaller, more physically robust, and faster

instrumentation while also consuming less power, reagents and calibrants. A lab on a chip, LED-based colorimetric system was combined with an inline filter and syringe pumps to detect an environmental sample and auto-calibrate with the use of on board standards. The automated colorimetric sensor was applied to nitrite and nitrate determinations during an unmanned 26-day field deployment in an estuarine environment (Beaton et al., 2012). The analytical performance, ruggedness, and specifications of the instrument exhibit the characteristics required for the practical application of field-ready microfluidic water analysis devices (Kovarik et al., 2012). The reported LOD of 15 nanomolar (Beaton et al., 2011) also represents a highly sensitive nitrite analysis which when applied to the study revealed fluctuations and patterns in the estuary which would have gone undetected under a traditional, manual monitoring regimen.

A series of in situ instruments from Systea Inc. (Italy) that have undergone prolific field deployment includes the Nutrients Probe Analyzer (NPA), Deep-sea Probe Analyzer (DPA), and Water In Situ Analyzer (WIZ) for autonomous and continuous environmental monitoring. Wet chemistry fluorimetric and absorbance based spectrophotometry analysis is carried out within a micro Loop Flow Analysis (μLFA) reactor which effectively removes air bubbles and enables sequential batch analysis and manipulates the fluidic components for reaction, detection, wash, and calibration cycles (Bodini, et al., 2015). The probes have been deployed on coastal marine buoys and platforms and have autonomously collected and reported measurements of nitrite, nitrate, orthophosphate, and ammonia. Reagents are stable for 3-4 weeks, and the units may be controlled through cellular communication (Azzaro and Galleta, 2006). Reports of Systea instrumentation documenting in-field use and instrument intercomparsions (Moscetta et al., 2009), continuous online measurements in a time series monitoring station (Grunwald, et al., 2007; Reuter et al., 2009), use at 1,500 meters depth (Moscetta et al., 2009), tethered to an

unmanned platform powered by solar and wind energy (Gunatilaka et al., 2009), and in automated sensor networks spanning multiple years of operation in open natural waters (Vuillemin et al., 2009; Bodini et al., 2015). The probes have been able to discriminate between low levels of nitrite (sub-µg/L) in oligotrophic waters, have withstood exposure in coastal waters for weeks at a time (Azzaro 2013; Vuillemin et al., 2009), and have correlated well with grab samples analyzed ex situ under laboratory conditions (Bodini et al., 2015). Systea has directly addressed ACT survey group (Figure 2) desires by making improvements in these areas and by providing constant technical support and assistance; in their view, this level of customer support, ease of calibration and maintenance will be important steps in the commercial strategy for nutrient analyzers.

One of SubChemSystems' current FDS, the Autonomous Profiling Nutrient Analyzer (APNA), is specifically designed to measure sub-meter scale nutrient gradients and can be deployed from a variety of platforms, both towed or stationary, and in continuous or autonomous data collection modes (Hanson, 2000). SubChemSystems' instrumentation has been reported to track nitrite and other nutrients in chemical plumes (Hanson and Moore, 2001), investigate thin plankton layers and the influence of stratification and turbulence, identify fine-scale structures of nitrite profiles, and investigate biogeochemical cycling as at ecologically critical scales for model validation and support (Hanson, 2000; Egli et al., 2009). Using the APNA in estuarine environments, Egli et al. (2009) captured changes in nitrite concentrations caused by tidal oscillations and advective fluxes, turbulence from coastal storms, upwelling, and freshwater runoff. Gilbert et al. (2013) sampled a salt-water estuary at an hourly time scale and used the enhanced spatial and temporal coverage to assess mixing behavior associated with ebb and flood

tides and demonstrated nutrient transformations and water body mixing across the tidal and seasonal cycles.

Sensors utilizing direct UV spectroscopy in analytical chemistry date back to the 1950s, and probes for nitrate are now industry standard in environmental monitoring because of the enhanced mathematical processing power afforded by miniature diode array detectors and microprocessors (van den Broeke, 2007; Sandford et al., 2007). S::CAN and TriOS have taken advantage of these advances to develop and produce instruments capable of indirectly measuring nitrite with chemometric models (Table 2). The S::CAN Spectro::lyzer uses a principal component analysis and partial least squares regression to create a multi-wavelength algorithm to construct calibrations, or 'spectral fingerprints,' to differentiate between chemical parameters. Such detection systems offer the advantages of robust and compact analyzers that can be placed directly in the sample media and require no moving parts or chemicals for measurement or cleaning (Rieger et al., 2004; van den Broeke et al., 2006; van den Broeke, 2007). Multiple optical path lengths give a range of sensitivity options, and with split-beam measurements coupled with multi-wavelength algorithms can compensate for turbidity, allowing these instruments to operate in a wide range of water types from industrial to ultra-pure (Rieger et al., 2004; van den Broeke et al., 2006). The Spectro::lyzer has been used to detect nitrite in water and wastewater treatment applications to provide relevant and appropriate controls of denitrification and aeration processes (Boley and Müller, 2004; van den Broeke et al., 2006), and for environmental river monitoring programs and industrial applications as early warning detection systems of organic contaminants (Libovic et al., 2006). Sandford et al. (2007) reported the use of the TriOS ProPS to investigate nitrogen cycling in natural waters and quantified diurnal nitrate and nitrite processes, patterns, and baseline perturbations.

Table 2. Examples of state of the art FDS for nitrite. Part 1. Data specifications.

Provider/ Instrument Name	ME GRISARD GmbH Water-APP 4004	Systea S.p.A. WIZ Probe	National Oceanography Center, UK	SubChemSystems Inc. APNA	S::CAN GmbH Spectro::lyzer	TriOS GmbH OPUS UV
Detection Principle	Wet Chemistry - Based on Griess assay	Wet Chemistry - Based on Griess assay	Wet Chemistry - Based on Griess assay	Wet Chemistry - Based on Griess assay	UV Spectroscopy - Indirect Chemometric Model	UV Spectroscopy - Indirect Chemometric Model
Monitored Parameters	NH4, NO2, NO3, PO4, SiO4, Metals, Others	N-NH3, P-PO4, N-(NO3 + NO2); N-NO2	NO3, NO2	NO2, NO3, PO4, SiO4, Fe(III), NH4	NO3-N, NO2-N, COD, BOD, TOC, DOC, UV254, BTX, AOC, temp., press.	NO3-N, NO2-N, COD, BOD, TOC, DOC, NH2Cl, HS, temp.
Detection Limit and Range	Up to 40 mg/L	0.002 - 0.25 mg/L NO2-N (0.14 µM - 17.84 µM)	0.02 µM	1 cm: 0.05-50 µM; 5 cm: 0.05-11 µM	0 - 2.9 mM SW; 0 - 35.7 mM WW; Typical LOD 7.1 µM	0 - 14.3 mM NO2-N
Reported Accuracy	< 2%; +/- 2% of calibration value	+/- 2% accuracy at 100%; +/- 3% accuracy at 5%	NA	NA	NA	NA
Reported Precision	NA	+/- 2% accuracy at 100%; +/- 3% accuracy at 5%	Estimated Uncertainty (0.08 µM, 2x Stdev)	2% (of range)	NA	NA
Interference Compensation and Sample Pretreatment	Filter module available	DTPA and TRIS buffer; UV-digester; 0.45 um filtration cartridge option; Copper-based antifouling protection; Full removal of air bubbles	0.45 µm pore size Millex HP inline filter; Reference detector corrects for background absorption	4-filter sampling head (10 µm); Copper mesh surrounding each filter; Reference detector corrects for background absorption	Spectral deconvolution algorithms	Spectral deconvolution algorithms
Minimum Temporal Resolution	4 readings / hr.	30 min. for a full 4 parameter cycle	5 min.	1/sec. (~7days duration)	20 sec.	< 1 min.

Key: D=Diameter H=Height SB=Standby Mode OP=Operational Mode Press=Pressure Temp=Temperature Avg= Average Stdev= Standard Deviation SSW= Surface Seawaters IW= Inland Waters DW=Drinking Water WW=Wastewater GW=Groundwater

Table 2 Continued. Part 2. Operational considerations.

Ambient operating conditions	SSW, IW; 5 - 40° C; Up to 7 m depth; Range of salinities	SSW; 4 - 40° C	SSW; Operated in 4 °C without impact; Has descended to 170 m depth	SSW; Up to 200 m depth, press. compensated	SSW, GW, DW, WW, IW; Operating press.: 0 - 3 bar; Storage temp.: -10 - 50° C	SSW, GW, DW, WW, IW; 300 m depth; Operating temp.: 0 - 40° C
Weight	Weight in air, ready for operation: 8 kg	8 kg in air (without reagents)	1.1 kg in water (no battery), 1.5 kg (with battery)	Air: 8.8 kg; Water: 1.4 kg (7.5 L displacement)	3.4 kg (including cable)	NA
Dimensions	NA	Analytical unit: 140 mm D x 520 mm H; Reagents container: 70 mm D x 200 mm H	100 mm D x 200 mm H (without reagents or power supply)	16.8 cm D x 32.6 cm H	44 mm D x 612/656 mm H	48 mm D x 460 mm H (without connector)
Power Supply	12 VDC External Battery	12 VDC, 3 A; 5 m underwater cable or portable battery pack with photovoltaic cell	NA	12 VDC/ 12-75 VDC; Single underwater cable	11 - 15 VDC; Can be supplied by solar power	9 - 28 VDC; Optional external battery pack
Power/ Electrical Consumption	Avg. power consumption: 1.2 W; SB: 0.063 A; OP: 0.1 A; With heater: 1.7 A	OP: 6 W; SB: 3 W; Maximum 1 A	1.5 W; 1200 J per sample	OP: 28 W; SB: 33 µW	Avg.: 4.2 W; Max: 20 W	4 - 20 mA
Reagent Life	NA	4 - 10 weeks; cooling by ambient water	Min. 1 week; Max. up to several months (reagents stored separately)	~3 months	NA	NA
Reagent Consumption	< 0.5 mL/measurement	30 - 60 µL per analysis; minimum 1,000 analyses on board	In continuous operation: 0.088 L reagent and 0.029 L standard per 24-hr period	NA	NA	NA
Data Output & Telemetry	RS232 serial port; Remote control possible	RS232 serial port; Telemetry via cellular - GSM modem; WiFi capable	RS232 serial port	RS232/485-ACII, Ethernet; Telemetry via RF/cellular/Wi-Fi/Acoustic/Iridium/LAN	Integrates into family of S::CAN sensors and control systems; Data logger mode possible; Telemetry possible with additional components	RS232/485, various protocols; external data logging possible; Telemetry via network TCP/IP

Table 2 Continued. Part 3. Complimentary considerations.

Commercially Available	Y	Y	N	Y	Y	Y
Capital Cost/Unit	NA	NA	NA	NA	NA	NA
Notes on Autonomy	Automatic change of light path length; Maintenance free time at least 8 weeks	Max. 2 months autonomy; auto-calibration and wash cycles	Capable of 6 blank corrections per hr; Standard curve up to 4 standards	5-point calibration by standard addition method	Maintenance free operation - no moving parts, no consumables; Automatic cleaning with compressed air	Maintenance free operation - no moving parts, no consumables; Automatic cleaning with compressed air
Reported Deployment Modes & Geographic Areas	NA	Moored buoy systems; Coastal waters, lagoons, oligotrophic waters	CTD, benthic lander, buoy; Estuary; arctic open waters, seafloor oxygen minimum zone	Vertical or towed profilers, autonomous moored profilers, autonomous underwater vehicles, fixed-depth piers or moored buoys; Coastal waters, rivers and estuaries	Mounting and measurement directly in the media or in a flow cell (monitoring station); WWTP	Mounting and measurement directly in the media or in a flow cell accessory
Consecutive Time Reported In Situ: Autonomous Nitrite Measurement	NA	14 days; 18 days; 35 days (typhoon limited)	57 hrs; 70 hrs; 26 days; 40 hrs	3 weeks; 19 days; 23 day period (non-consecutive)	30 days	NA
Key References	ME GRISARD GmbH; ACT	Bodini et al., 2015; Moscetta et al., 2009; Vuillemin et al., 2009; Systea S.p.A.; Vuillemin and Sanfilippo, 2010	Beaton et al., 2011, 2012; Yücel et al., 2015; Cross et al., 2015	Egli et al., 2009; Hanson 2000; Hanson and Moore, 2001; Gilbert et al., 2013; SubChemSystems, Inc.	S::CAN GmbH; Rieger et al., 2004; Boley and Müller 2004; van den Broeke et al., 2006; van den Broeke, 2007	TriOS GmbH; Sandford et al., 2007; Pellerin et al., 2013

1.2. Discussion

1.2.1. FDS Outlook: Technical advancements and limitations

The examples of nitrite FDS listed in Table 2 represent a culmination of the FDS vision that has been in the making for over four decades. The newest generation of this technology has begun to make the necessary adjustments for long-term, unmanned deployments outlined by Campos and da Silva (2013). Power requirements, reagent consumption, and size have all decreased while robustness, variety of instrumentation, and telemetry capabilities have increased. For nitrite analysis, wet chemistry analyzers with the Griess reagents have proven to be chemically robust across large salinity and temperature gradients. Sample introduction and pretreatment requirements using 10 µm screen filters, at times paired with copper wire for biofouling mitigation, have proven to be effective with minimal maintenance in real-world deployments (Egli et al., 2009; Vuillemin and Sanfilippo, 2010). Instrument service to components, including cleaning of external films and particulate matter, and replacement of reagents, standards, wash solution, and filters have been reported to take place on a weekly or bi-weekly basis, especially in turbid and highly eutrophic environments. Cleaning cycles and compressed air backflushing can also serve to increase deployment life (Table 2).

UV spectrophotometers are not limited by the factors affecting the reliability of wet-chemistry analyzers, but their sensitivity and accuracy is inherently limited by dependence on the ability of statistical models to keep pace with changes in the sample matrix (Winkler et al., 2008). In oligotrophic conditions, UV spectrophotometers have not adapted well to discern trace levels of nutrients (Vuillemin and Sanfilippo, 2010). Though the sensors have the ability to be programmed with local, on-site calibration, in some cases very poor correlation with standard methods have been experienced (Boley and Müller, 2004) and even site-specific calibration

algorithms may not be sufficient to account for particle and turbidity disturbances (Winkler et al., 2008; Rieger et al., 2004), leaving these type of sensors better suited as screening or alarm tools (van den Broeke et al., 2006).

Electrochemical sensors for nitrite meanwhile are advancing towards autonomous, long-term in situ deployments and are improving their figures of analytical merit. Electrochemical techniques have been coupled with biosensors in commercial units (UniSense, 2016) and have made advancements in mass production techniques by screen-printing electrodes, furthering the vision of low-cost, reagentless, and even disposable sensors (Radu et al., 2013). Before electrochemical sensors for nitrite can effectively integrate into FDS instrument packages and penetrate commercial markets, developers must resolve maintenance and stability issues that preclude electrochemical FDS from long-term, autonomous in situ operation. Currently, the high frequency of cleaning, recalibration, and electrode rejuvenation or membrane replacement prevents long-term deployments (Bende-Michl and Hairsine, 2009; Campos and da Silva, 2013). Lack of stability and robustness of the reference electrode also represents a hurdle, as common electrical fluctuations and drifting upon continuous sample exposure and less than ideal operating conditions leads to poor reproducibility, accuracy, and sample carry-over (Campos and da Silva, 2013; Radu et al., 2013; Yilong et al., 2015; De Marco et al., 2007). One solution involves pairing electrodes with fluidic devices and applying FIA techniques to mitigate electrode fouling and drift, clean electrodes, desorb foulants, and extend operational lifetimes (Tossanaitada et al., 2012; De Marco et al., 2007).

1.2.2. Data handling, quality assurance, standardization, and nomenclature

For all the progress being made and efforts devoted to the areas of FDS development, there is a disproportionate lack of literature surrounding the initiative to develop means of quality assurance, quality control, field experimental standardizations, metrics, and nomenclature that exists in laboratory analytical chemistry yet may differ entirely under in situ circumstances.

In 1978, Koeppen et al. recommended efforts be made to establish uniform performance criteria, standardized performance specifications (from sources other than the manufacturer), a centralized information system listing commercially available instruments and development projects along with performance test data, well-defined and comprehensive classification schemes for instrumentation, and means of verifying performance against standard criteria (Koeppen et al., 1978a,b.) In an assessment of the US government's technology for oceanographic research and monitoring in 1981, the US Congress' Office of Technology Assessment also identified the issues surrounding data sharing and collection in oceanic systems. The data, which was collected at a great expense to the public, often went unused because of non-standardized formatting and difficulty retrieving standardized quality assurance from the original data producer (US OTA, 1981). A quarter of a century later, authors still found documentation and agreement on test protocols, international standards, and stringent metrics lacking (Mowlem et al., 2008; Guntilaka and Dreher, 2003).

As can been seen in Table 2, categories of tabulated data on FDS specifications are not always easy to compare as measurement units and performance parameters are often inconsistently reported. To make matters more difficult, there exists no defined methodology for reporting performance specifications in operational environments. Developers and users of the nitrite FDS reviewed have at times validated their data against spot measurements using grab

samples and traditional laboratory analysis; however, this is not always the case and there are no requirements for how to consistently sample, measure, and report. Consumers are not always provided the circumstances under which performance measures were taken, making assessment of in situ performance characteristics difficult.

There have been encouraging signs from organizations such as the ACT, which has generated performance evaluations, workshop reports, and survey documents on FDS sue and performance. FDS users and developers have also creatively integrated auxiliary physical and chemical probes (e.g. CTD, optical backscatter, DO, PAR, Chlorophyll a, CDOM, etc.) to make measurements that can lead to inferences about the quality and trends of nutrient concentrations. Yücel et al. (2015) used the raw photodiode output from the detector as internal calibration check, and Gilbert et al. (2013) developed a MatLab program to monitor data quality and flag 'bad' readings by using the software to identify baseline, sample, and calibration peaks (standard addition method) and supported the data with manual grab samples. As Prien (2007a) has noted, even if accuracy suffers at times, the ability of FDS to reveal overall trends and patterns may be a bigger determinant of their usefulness.

To achieve practical employment and pervasive value of FDS, associated data products must be interoperable, discoverable, traceable, easily interpreted, and standardized. Developers can design more effectively and efficiently by targeting the instrument to specific applications (Ríos et al., 2012; Kovarik et al., 2012) within a complete product life cycle (Figure 4). Considerations and decisions made early on in the design process regarding target performance ranges and specifications (Table 1), user needs (Figure 2), production management (e.g. lean, agile, concurrent engineering), device implementation and disposal have significant impacts downstream. A critical undertaking necessary for success of FDS involves instrumentation

developed with interoperable features and interfaces, and integrated with automated QA/QC associated procedures (Pellerin et al., 2016). Table 3 provides examples of such data management practices.

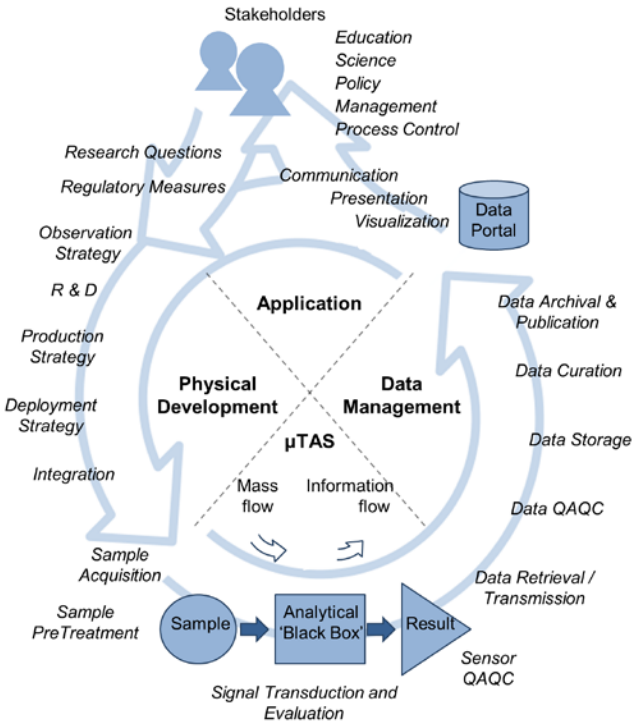

Figure 4. FDS and data product life cycle. FDS operates a µTAS to turn a physical/ or chemical attribute into an information product that is processed and disseminated.

Table 3. Relevant examples of FDS and data product life stages.

		Relevant Water-Centric Examples	Reference
Application/Parameters		See Table 1; Figure 2	
Physical Development: Marine Sensing Network		ARGO Program	Ifremer NA-ARC
Nutrient µTAS: FDS		See Table 2	
Data Management	Validation/ QAQC Procedures & Codes	IOOS Manual for Real-Time Quality Control of Dissolved Nutrients Observations	Willis et al., 2015
		NDBC Handbook of Automated Data Quality Control Checks and Procedures	NDBC, 2009
		Guidelines for Optical Techniques for Determination of Nitrate in Environmental Waters.	Pellerin et al., 2013
		Manual of Quality Control Procedures for Validation of Oceanographic Data	IOC/IODE 1993
		WOCE Operations Manual	WOCE, 1994
	Interface Standards/ Data Protocols	OGC Sensor Model Language	Botts et al., 2014
		OGC Sensor Observation Service	Broring et al., 2012
		OGC Sensor Planning Service Interface Standard	Simonis et al., 2011
		Observations and Measurements Conceptual Model - XML Implementation	Cox, 2011
	Data Management and Planning	Data Elements for Reporting Water Quality Monitoring Results	NWQMC, 2006
		NSF Data Management Plan Overview	CUAHSI (b)
		Strategic Plan for Coastal GOOS	GOOS, 2012
	Data Exchange Platforms	Water Data Center	CUAHSI (a)
		European Commission Information Exchange Platform and Library	WFD CIRCABC
		OGC WaterML 2.0	OGC
		Water Quality Portal	NWQMC
		ACT Technologies Database & Evaluations	ACT

1.2.3. Technological evolution

The FDS listed in Table 2 have demonstrated the possible applications for such technology. The information produced by these sensors can be powerful information for testing hydrodynamic models, validation of remote sensing products, as risk assessment tools for hazardous pollution events and plankton blooms, and as the basis for planning ship-based campaigns. The more descriptive snapshots of concentrations across time and space can greatly improve and increase our understanding of biogeochemical processes and nutrient transformations and pathways, verify models, and save costly resources. The provision of horizontal mapping and vertical profiling, time series measurements, and daily cycling in dynamic systems can capture both episodic events and reveal patterns of hydrological, environmental, and human influences.

The information surrounding water quality will become more important if both regulatory measures and the general public's concern for environment health and resource management increases. This is already evidenced by a growing number of firms offering or developing FDS along with political manifestations such as the European Water Directive Framework generating funding for WSN, the ACT's Nutrient Sensor Challenge, and the impetus to tackle pollution which led the People's Republic of China's to invest in over 100 Systea Probes to monitor the South China Sea (Bodini et al., 2015).

The information provided by FDS must also evolve in tandem with the technological evolution from custom, or purpose-built sensors that fill a role the market has not yet met or cannot fill, to a product that has adapted to meet customer needs and has added economic value (Figure 1). In order to become a true commodity, the information that FDS provides as a product must be standardized and organized. Only then can it become a true utility to researchers and

resource managers. To achieve this goal, we must develop a framework to properly analyze and discuss FDS performance in operational environments. This framework can fully support and promote their implementation. Issues such as sensor inter-calibration that could jeopardize an entire experiment could instead be solved efficiently with tools built to handle massive data check procedures. These tools could open the door for new ways to visualize, analyze, and disseminate complex data sets (Vuillemin et al., 2009).

In the Nutrient Sensor Challenge and Preliminary Market Outlook (2015), the ACT has identified a crucial tenth TRL that involves diffusion and expansion and occurs after early adoption and growth of primary markets by early adopters. The ACT desires a new generation of nutrient sensor that can overcome cost prohibitions, error and uncertainty issues, calibration errors, network communication losses, and overall uncertainty in underlying parameters. The organization has outlined an aggressive time to market and rate of adoption over the next decade (ACT 2015). A case study by Hanna et al., (2015) on innovation timelines found that the average time taken for new technologies to reach widespread commercialization is around 40 years. As we approach 40 years since Koeppen et al. (1978a, b) made recommendations to NOAA for developing nutrient FDS technology and evaluations, it is appropriate that we set up the support system FDS need in the form of standardized in-field performance metrics and specifications. This effort can help cross the innovation chasm by providing conservative and skeptical consumers the evidence they need to trust and accept FDS.

These objectives will require more frequent field deployment validation and inter-calibration between FDS and other analytical techniques (Allan et al., 2006). Furthermore, developers must consider the entire life cycle of the FDS concept and methods to deliver user-friendly and insightful instrumentation whose curated data products might encourage more

information gathering across sectors of government, industry, and academia. Consequently, developers must also consider plans and resources for handling the vast amounts of data that will increase with the scaling up of FDS (Campos and da Silva, 2013; Shade et al., 2009). Protocols to manage databases and provide quality assurance and quality control measures are challenging requirements that will need to be addressed in order to make automated sensing technologies optimized and useable (Shade et al., 2009).

1.3. Conclusion

The potential consequences of erroneous, unreliable, imprecise, and inaccurate data can exaggerate the uncertainty surrounding FDS. The associated risks can cause an underlying fear of failure that limits adoption and stymies innovation. Innovations, however, are rarely offered in ready-made packages, but more as opportunities that come with failure as a necessary ingredient (Ortt and Smits, 2006). Trial, error, and failure of FDS are essential processes in the discovery of new ideas and improvements. Though acceptance, legitimacy, and fiscal concerns threaten the leap forward for FDS, Williams (2011) has reminded the scientific community that the consequences of failing to innovate and transcend technological lapses can have very dangerous consequences leading to the loss of scientific knowledge and understanding, ultimately resulting in the inability to adapt to a changing world. To truly innovate the uses of FDS for nutrients, researchers and resource managers will need to learn manage risks, overcome traditional views surrounding monitoring and analytical paradigms, and embrace the idea of trial and error on the way. It is the aim of this review to encourage researchers and resource managers to approach FDS with this mindset in answer to the Grand Challenge.

FDS for nitrite and nutrients in general have made significant and notable advancements since the time of Grand Challenge. Much of this we know thanks to the reports available in peer-reviewed journals and conference presentations documenting instrument development and performance in actual field deployments. Of course, for every success story, there are deployments that do not go as planned and remain undocumented. We encourage those developing and implementing FDS, the innovators, the visionaries, and early adopters to continue to report FDS uses and experiments under all circumstances and operational environments to add to our growing body of knowledge on the subject, indeed on the science of this technological application.

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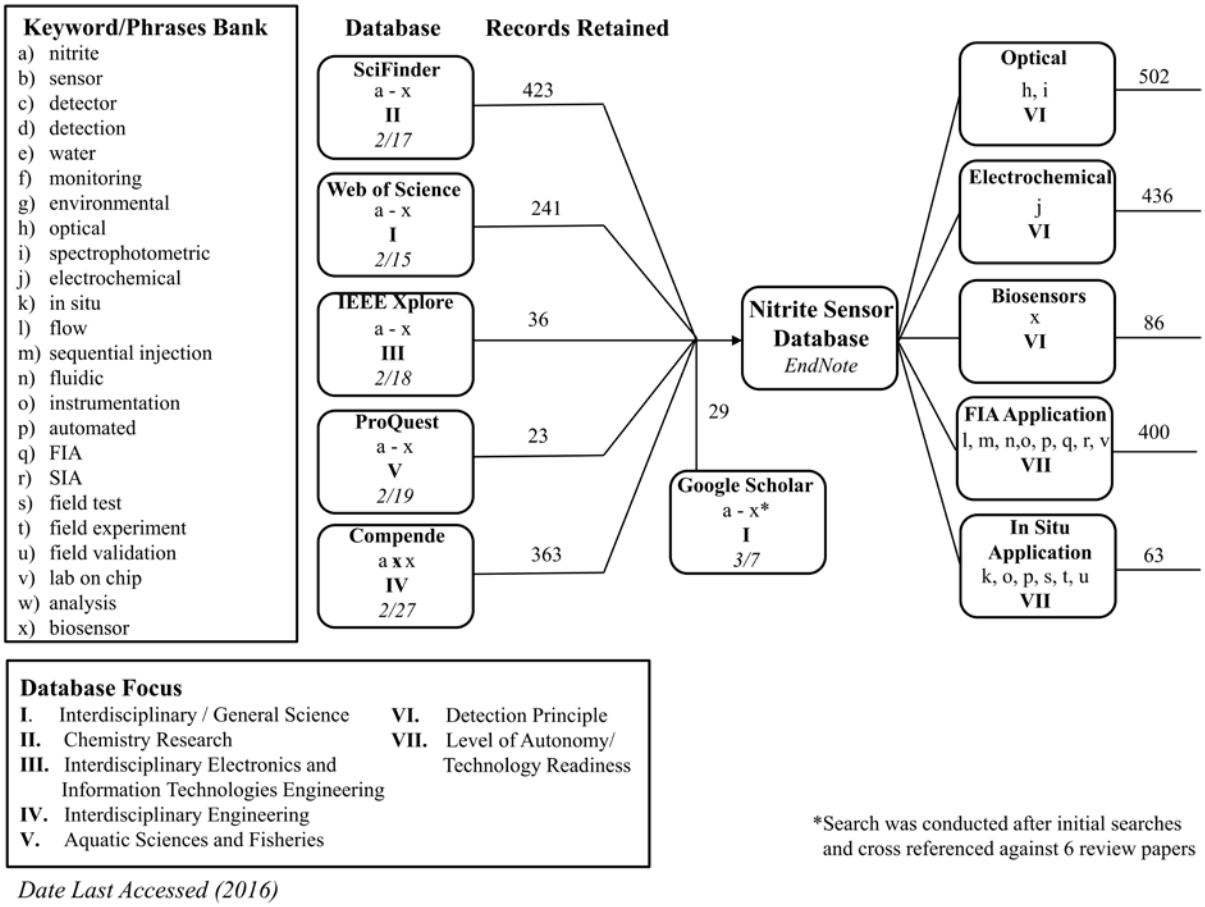
1.5. Appendix A

Figure A.1. Keyword search flow chart for literature analysis. Literature searches were performed over the period of 2/3/16 to 3/7/16.



2. Chapter II

Development of a simple and configurable fluidic system for the detection of nitrite in aqueous samples

Abstract

This work describes the development of an automated detection system for determination of nitrite using inexpensive and commercially available components and chemistry. While nitrite analyzers have begun to penetrate markets worldwide, generally speaking they are not widely implemented or practically adopted. We report the design and testing of a prototype module that uses a direct and simple measurement method built on proven fluidic injection analysis and colorimetric techniques. The opto-fluidic system demonstrated appreciable precision (relative standard deviation <2.0%), sensitivity (limit of detection <0.2 μM NO_2^-), and linearity (R^2=0.999) over a relevant linear range (0-25 μM) in under 30 minutes and for around US$1,000. The system was validated against a US EPA standard method for nitrite on a commercial spectrophotometer and autoanalyzer. The prototype is based on the programmable Arduino microcontroller, and is easily configurable for additional sensitivities and colorimetric assays. The analyzer presents potential for use as a low cost, adaptable, and readily accessible instrument for unattended monitoring in process control applications such as aquaculture operations.

2.1. Introduction

The automation of analytical methods as chemical 'Total Analytical Systems' for point of use or in situ detection and quantification of nitrite and other nitrogenous compounds has been an active area of research for over 20 years. The motivations to develop these unattended monitoring technologies reside in their potential to obtain high resolution data over prolonged periods with a simultaneous reduction in cost of labor and sampling infrastructure (Ho et al., 2005; Johnson et al., 2007; Prien et al., 2007; Pellerin et al., 2016). Early innovations focused on automated flow injection analysis (FIA) coupled with spectrophotometry packaged in field deployable and submersible fit-for purpose research instruments (Daniel et al., 1995; David et al., 1998; Byrne et al., 1999; Le Bris et al., 2000; Masserini and Fanning, 2000; Thouron et al., 2003). Variations of FIA techniques developed as part of benchtop prototypes (Gabriel et al., 1998; Greenway et al., 1999; Rocha and Reis, 2000; Petsul et al., 2001; Legnerová et al., 2002; Hirata et al., 2003) demonstrated advantages such as improved precision, fast response and low reagent consumption and waste generation compared to manual methods.

More recent advances have integrated fluidic and detection systems into lab on a chip devices (da Rocha et al., 2012; Czugala et al., 2013a,b; Horstkotte et al., 2013; Hwang et al., 2013) that further reduced analysis times and reaction volumes (to µL and nL) and improved precision (Ríos et al., 2012; Worsfold et al., 2013; Antony et al., 2014). These efforts have culminated in researchers' development of sensor packages capable of autonomous operation for measurements of nutrients for weeks at a time (Diamond et al., 2011) and tested in real-world environments for nitrite/nitrate (Beaton et al., 2011, 2012; Cogan et al., 2015), phosphate (Cleary et al., 2008), and ammonia (Cogan et al., 2014). Some of these systems have broken through the

research and development barrier and are entering the market as commercially available high-frequency analyzers (i.e., APNA, WIZ, NAS-3, EcoLAB).

Improvements in developer grade microelectronics have seen a large expansion and availability of prototyping tools for embedded systems. Consumer accessible microcontrollers and single board computers such as the Arduino, Raspberry Pi, and BeagleBone have been the catalysts for the development and large scale commercialization of an array of microelectronic subsystems that include actuators, communications, optoelectronics and data storage. In addition to this inexpensive and often open source hardware, a large crowd-sourced and on-line user base and support network exists that enables a bottom-up development approach (Hagel et al., 2010). For research applications this provides individual scientists the ability to rapidly design, build, and test devices without the complete reliance on scientific instrument manufacturers or application engineers (Von Hippel, 2005).

While the application of microfluidic and lab on chip technologies represents the current trend and gold standard in the automation of analytical chemistries, their fabrication is reliant on specialized equipment that is out of the reach of many laboratories. Additionally, once manufactured, many of these devices are not easily configured for applications other than their intended purpose and still require a degree of modularity for real world application (Campos and da Silva, 2013). Here we report the development of a readily configurable opto-fluidic system that uses primarily off-the-shelf, low cost components and demonstrate its use as an automated chemical sensor for nitrite. All components are readily replaceable, which offers the advantage of enabling the user to clean or replace components if they become fouled or contaminated. Additionally, components can be easily interchanged for applications that require increased chemical compatibility or reaction sensitivity. As the control electronics are based on an open

source Arduino microcontroller, software can be readily developed for alternative applications. These system attributes are particularly beneficial for rapidly developing automated analytical assays in a research environment, while maintaining a simple fluidic design enables end users with minimal training to operate and maintain the instrument.

The aim of this study was to develop an inexpensive, easy to use automated system for nitrite analysis that could be applied as a process control tool in the aquaculture industry, where incomplete oxidation of ammonia from fish waste or excess food can lead to elevated nitrite levels and can cause methaemoglobinemia, or 'brown blood disease' (Durborow et al., 1997; Buttner et al., 1993; Kroupova et al., 2005). Traditional bench top assays and semi-quantitative tests (e.g., test strips and color comparators) remain the most heavily relied on methods in the aquaculture industry for nitrite monitoring, though use of FIA and automated sensors for aquaculture management has been supported for some time (Ariza et al., 1992; Fowler et al., 1994; Badiola et al., 2012). Multiple test kits (e.g., Hach, Api, LaMotte, etc.) for nitrite are commercially available; however, their accuracy and sensitivity may be severely compromised due to inter-user variability and the semi-quantitative nature of the tests (Ormaza-González and Villalba-Flor, 1994). Simplified portable and handheld spectrophotometers quantify concentration more accurately than test strips and test kits, but still require analyst operation to retrieve samples and to operate, clean, and calibrate instrumentation.

Currently there are excellent examples of automated nitrite analyzers for use in the environment (Egli et al., 2009; Gilbert et al., 2013; Bodini et al, 2015; Yucel et al., 2015), along with significant challenges and adjustments for size, power, storage, and ruggedness requirements for prolonged deployment in real world conditions (Campos and da Silva, 2013). A sensor targeted for use in an application such as aquaculture can utilize the concepts proven by

such instrumentation, and benefit from a more controlled setting with considerably less demands for analytical operation, maintenance, and cost. This study investigates design and performance considerations, including the assessment of a number of commercially available reagents, for a simple, automated, and inexpensive system capable of producing nitrite measurements with relevant sensitivity for an aquaculture setting.

2.2. Experimental

2.2.1. Reagent and standard preparation

The Griess reagent was prepared according to the American Public Health Association (APHA) standard method 4500 NO_2^- B (Eaton et al., 2005) with analytical grade chemicals and type 1 ultra-pure water (18.2 MΩ-cm resistivity, ELGA, USA). Commercial nitrite reagents were purchased as part of off-the-shelf test kits (Table 4) and used in accordance with the manufacturers' instructions. The manufacturers of the API and ELOS reagent solutions did not disclose peak absorbance wavelengths, so both chemistries were tested by scanning absorbance across the visible spectrum (400-700 nm) up to concentrations of 10 µM NO_2^- (Figures B.1a, b and B.2a,b in Appendix B).

Nitrite standards used for general instrument development were created by dissolving 6.9 g of sodium nitrite (Sigma-Aldrich, USA) into 1 L of type 1 ultra-pure water. The 100 mM stock solution was diluted further with ultra-pure water to create standards for testing absorbance measurements. Additional nitrite standards used for standard curve construction, reagent shelf life, and instrument validation procedures were prepared by serially diluting a 1,000 ppm (0.02 M) certified concentrated stock solution (Fisher Scientific, USA) with ultra-pure water in 100 mL volumetric flasks. For shelf life experiments, nitrite standards ranging from 0.25 – 25 µM

were prepared on the day of analysis from a 100 mM stock solution stored in an amber glass bottle at 4°C. A pre-mixed solution of Molecular Probes Griess reagents A and B was prepared in 1:1 v/v ratios according to the manufacturer's instructions and stored at room temperature in the dark for the course of the shelf life experiment. At weekly intervals, the pre-mixed Molecular Probes' reagent was compared to reagent stored according to the manufacturer's instructions using the Molecular Probes' spectrophotometric method on a Perkin Elmer Lambda 25 spectrophotometer (5 cm cuvette). During prototype testing, nitrite standards and reagents were stored in 50 mL Falcon polypropylene centrifuge tubes (Corning, USA), and 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, USA) respectively.

2.2.2. Instrument design

2.2.2.1. Optical detection system

Measurement of nitrite was based on the relation of light absorbance to reacted Greiss reagent described by the Beer-Lambert law (Equation 1). Absorbance can be measured as the base 10 log of inverse light transmission (Equation 2), and exhibits a positive linear relationship with increasing concentration.

$\boldsymbol{A = \varepsilon bc}$ where: $\boldsymbol{A}$ = Absorbance; $\boldsymbol{\varepsilon}$ = Molar absorptivity
$\boldsymbol{b}$ = Pathlength; $\boldsymbol{c}$ = Concentration **Eq. 1.**

$\boldsymbol{A = log_{10}\frac{P}{P_0}}$ where: $\boldsymbol{P_0}$ = Radiant power from the source
$\boldsymbol{P}$ = Radiant power transmitted by the sample **Eq. 2.**

Radiant power ($\boldsymbol{P_0}$) was supplied by a Super Bright Green LED (Kingbright, UK) with a peak wavelength at 526 nm and a spectral half-width of 30 nm. The LED was driven by a 20 mA constant current. Light intensity was detected using a TCS3200-DB light-to-frequency

Color Sensor Module (Parallax, USA) that consisted of an 8x8 photodiode array comprised of sets of 16 evenly distributed photodiodes each with a red, green, blue filter or unfiltered (clear) and a collimating lens to focus incoming light onto the detector array. For nitrite analysis, the output from the color sensor's green channel (~525 nM) was used to measure the peak absorbance wavelength of nitrite/reagent compound. All light readings were detected using 100% gain and an integration time of 500 ms.

The optical module consisted of interchangeable 10, 20 or 50 mm path length PEEK flow cells (FIALabs, USA). The LED and color sensor were secured onto the flow-cell optical ports using custom fabricated PVC adapters. Fluidic ports on the flow cells were fitted with 1/4"-28 UNF polypropylene adapters with 1/8" barbs (Eldon James, USA). The Z-style flow-cells were mounted with optical paths at an upward angle to help air bubbles escape.

2.2.2.2. Fluidic system and hydraulic control

The fluidic system was configured as a reverse flow injection analyzer (rFIA) and used a multicommutation approach to draw sample, inject reagent, mix, and move fluid through the flow-cell detector and out to waste (Figure 6a). Fluids were pumped with a speed-variable and reverse- flow enabled peristaltic pump driven by a 12 VDC, 150 mA, brush motor (APT Instruments, USA) and pulled through an upstream flow network constructed of 3 miniature 3-way switching miniature solenoid valves (Parker Hargraves, USA) that were actuated sequentially in a distributer mode to pass fluids (Figure 6a). The fluidic manifold was constructed of 1/8" [3.18 mm] ID Flexelene 135C FLXC1-2 tubing (Eldon James, USA) in a serpentine fashion to the flow-cell and out to the pump /waste (Figure 5). To enable the measurement of pump head rotations, 3 neodymium magnets were glued into existing holes in

the pump head assembly and a Honeywell SS315AT Hall Effect sensor was glued to the pump exterior. Calibration of pumped volumes was performed by taking the difference of mass (mg) of Type 1 water or reagent in a glass beaker before and after withdrawing either a.) A specified number of one-third pump roller rotations or b.) A specified number of milliseconds. The benchtop system was integrated into a custom platform built with 0.25 mm thick PVC sheets.

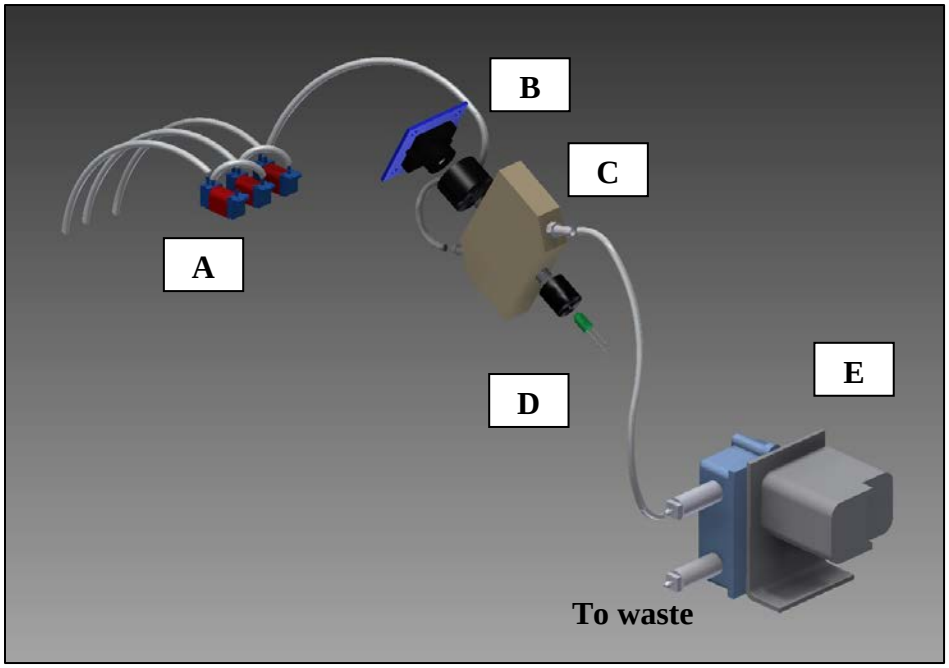
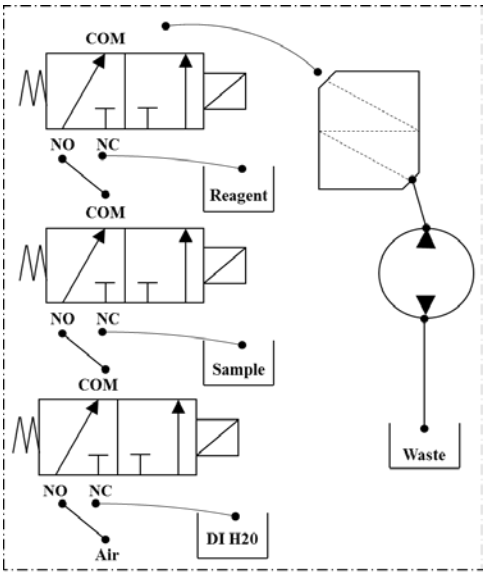


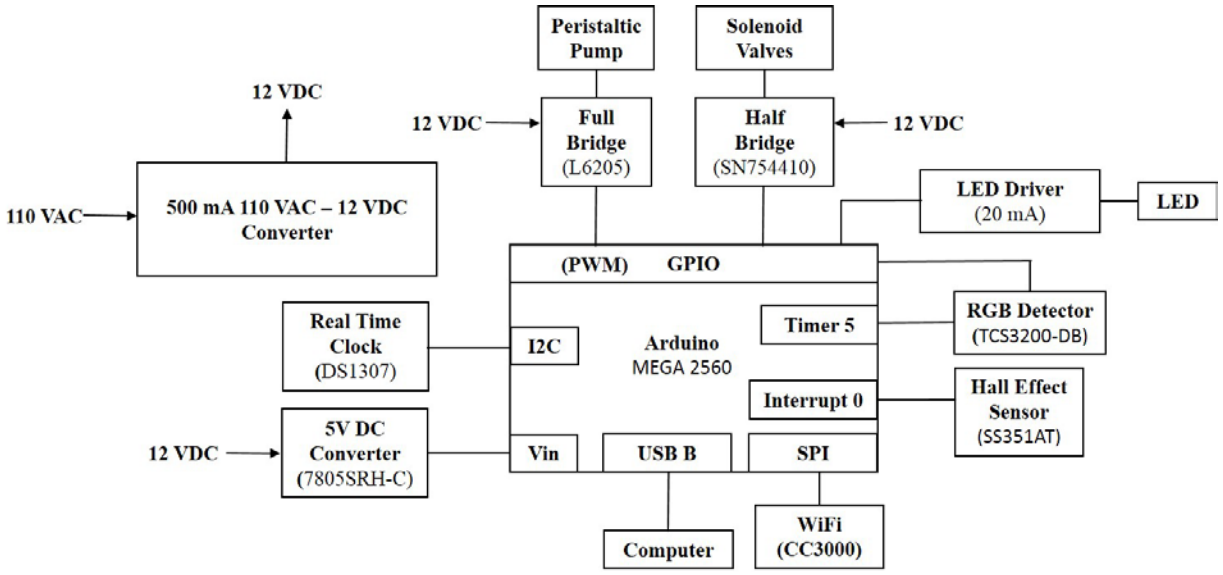
Figure 5. Illustration of prototype fluidic module. A. 3-way miniature solenoid valve array. B. Color sensor C. Flow-cell. D. Green LED E. Peristaltic pump.

2.2.2.3. Control electronics

The system was controlled by an Arduino Mega 2560 microcontroller. A custom designed and commercially available PCB board (ABL Controls, USA) was used to interface the 5 VDC logic of the microcontroller with the instruments' 12 VDC fluidic systems. The interface board contained a dual full bridge driver (STMicroelectronics, Switzerland) to enable forward and reverse actuation of the pump. Quadruple half bridge drivers (Texas Instruments, USA)



a.



b.

Figure 6. Instrument schematic. a. Hydraulic circuit diagram. **b**. Electronic block diagram.

were used for solenoid valve actuation. A DS1307 Real Time Clock performed time keeping. Circuit design for the system is shown in figure 6b. Power was supplied to the system using a 110 VAC to 12 VDC "wall wart" style power pack. Control software for the instrument was

written in the Arduino Integrated Development Environment (IDE). The calculation of sensor readings' mean and standard deviation was performed by the software as a data quality check. This information was used to flag readings that fell outside the range of statistical significance (>99.5% confidence). For nitrite measurements using the prototype detector, data was output through the Arduino IDE serial monitor as comma separated values and analyzed using Microsoft Excel.

2.2.2.4. Instrument automation

The analysis cycle was initiated by first rinsing the system with 3 alternating ~300 µL plugs of DI water and air ('Clean System,' Figure 7). The system was then flushed with ambient sample water. During this time, 45 discrete light intensity readings on the sample plug were taken and averaged to serve as $\boldsymbol{P_0}$ (Equation 2) in the calculation for the transmission. The sample water was then displaced by a small plug of air before a further 1 mL volume of the sample was introduced into the system. As the sample moved through the valve array, 15 µL of Griess reagent was injected into the middle of the passing sample stream (Figure 7). Passive mixing of the sample and reagent was achieved through the fluidic path by using tubing bends and changes in orifice sizes across the valve ports, fluidic adapters, and flow-cell channel.

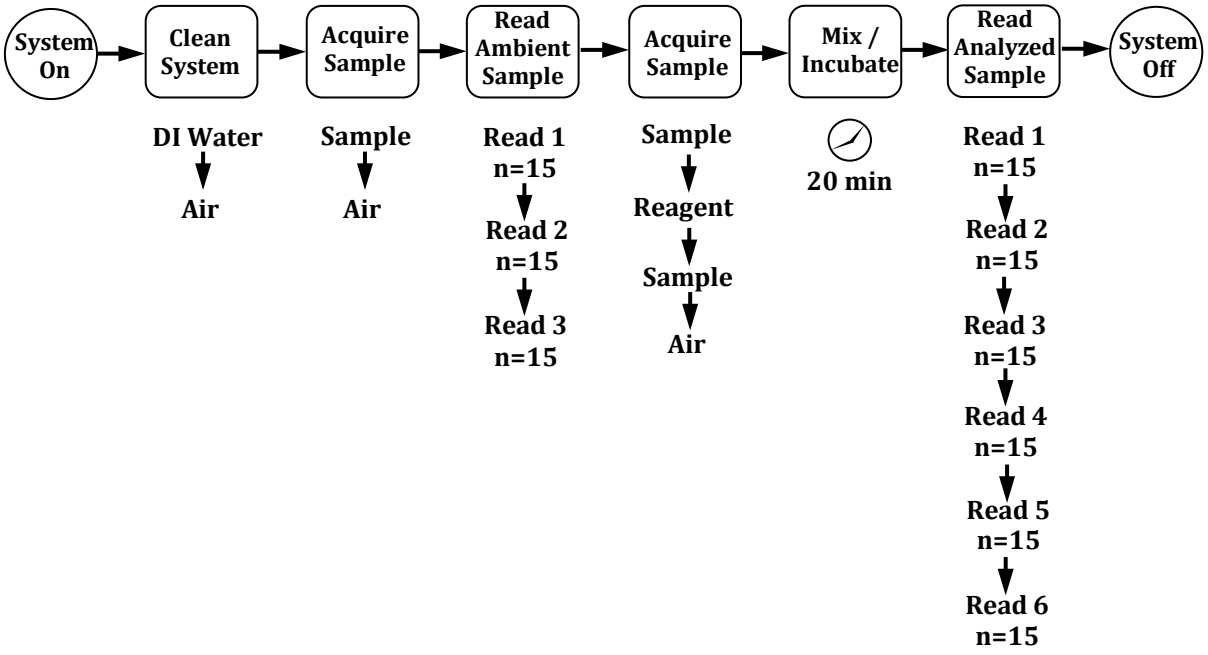


Figure 7. Process flow diagram. A sample plug is pumped to the flow-cell, where light intensity is measured (Read Ambient Sample) and serves as $\boldsymbol{P_0}$. After reagent injection and color development, light intensity is measured (Read Analyzed Sample) and serves as $\boldsymbol{P}$.

Active mixing was also performed by the pulsation effect from the peristaltic pump and by varying the pump's speed and direction (Hessel et al., 2005; Miro and Frenzel, 2004). After 20 minutes incubation time, the reaction plug was moved in and out the flow-cell 6 times at 70% of full pump speed, and resulting light intensity was measured and averaged over 15 readings that served as the final light transmission ($\boldsymbol{P}$) (n = 90, Figure 7). Absorbance was then calculated by the system using Equation 2.

2.3. Results and discussion

2.3.1. Reagent analysis

2.3.1.1. Reagent comparison

We assessed 4 commercially available nitrite chemistries (Table 4) for their ability to be integrated into an automated nitrite analyzer. Key considerations for this analysis were: 1) Potential for each method to be automated (e.g., liquid vs powdered reagents, number of reagents and fluidic manipulations); 2) Analytical performance (e.g., reaction time, correlation to standard methods); 3) Shelf life and storage considerations (e.g., refrigeration requirements, toxicity); 4) Commercial availability and cost per sample. The standard method using the APHA Griess reagent offered the highest sensitivity among the tested reagents with a calculated molar extinction coefficient (Ɛ) of 40,200 M^{-1} cm^{-1} (~ 87% of the theoretical Ɛ) (Hansen and Koroleff, 1999). Analytical performance of the commercially available reagents was also compared to this theoretical Ɛ value and ranked according to performance (Table 4). A theoretical Ɛ value for the ELOS test kit could not be calculated, as its response was not linear across a single peak absorption wavelength (Figure B.1a in Appendix B). The Hach NitriVer3 low-range nitrite reagent showed good sensitivity based on its molar extinction coefficient, however as it was composed of a powder that needed to be re-suspended before use it was deemed unsuitable for the proposed automated instrument and was not selected for further analysis.

Table 4: Comparison of commercially available nitrite reagents.

Protocol	Ɛ (M^{-1} cm^{-1})	λ (nm)	Incubation Time (min)	Linear Range	Cost/ sample (USD)	No. Reagents
Theoretical Griess assay[a]	~46,000[a]	540	20/1**	0-10 µM [0-0.46 ppm]	--	3
APHA Standard Method[d]	40,200	543	10	0.7-71* µM [0.03-3.2 ppm]	$0.07	3
Molecular Probes[e]	34,600	548	30	1-100* µM [0.05-4.60 ppm]	$0.26/ $0.05	2
API[f]	16,400	550	5	0-108 µM [0-5 ppm]	$0.05	1
ELOS[g]	NA	NA	10	0-43 µM [0-2 ppm]	$0.40	1
Hach	~29,900[b]	540	20	0-36 µM [0-1.64 ppm]	$0.37[c]	1

a. Hansen & Koroleff (1999).
b. Ormaza-Gonzalez & Villalba-Flor (1994).
c. Product # 2107169 (Hach, USA).
d. Eaton et al., 2005
e. Product # G-7921 (Molecular Probes, USA).
f. Product # 3317 (Mars Fishcare, USA).
g. Product # ELNO2 (ELOS, Italy).

*(with dilution in 1 cm cuvette)
**FIA method
ppm as NO_2^- ion

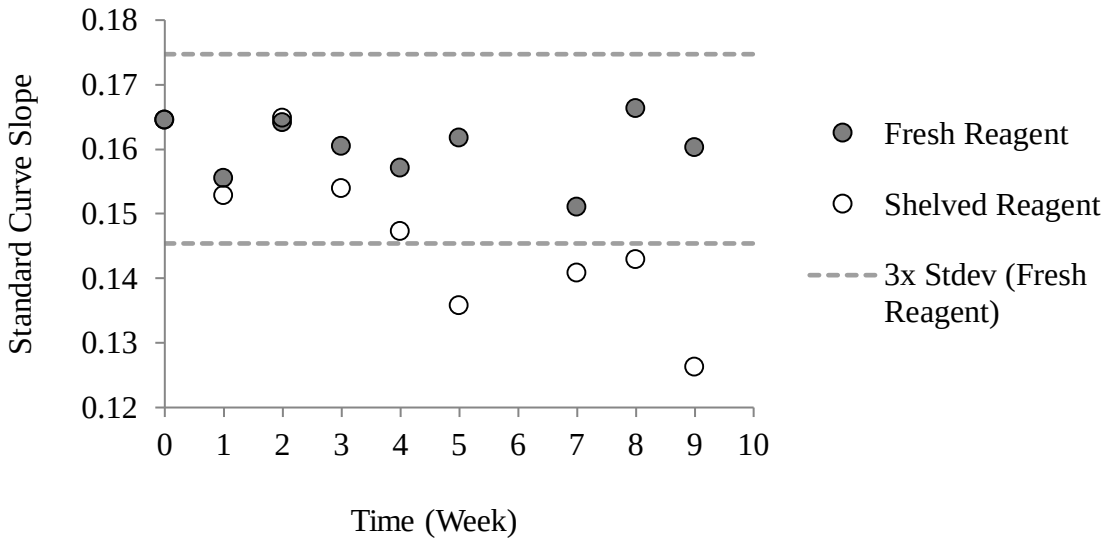
The Molecular Probes Griess kit is provided as a 2 reagent system and also contains a 1 mM sodium nitrite solution. Equal volumes of reagent A (*N*-(1-naphthyl)ethylenediamine dihydrochloride) and reagent B (sulfanilic acid) were mixed to form the Griess reagent. The Molecular Probes reagent was chosen as the highest rated chemistry because of its sensitivity, large linear range, and comparable price point per sample especially considering the small

volumes required for its microplate assay ($0.05 / sample). The API reagent was rated highest for simplicity and ease of use (1 liquid reagent, 5 minute incubation time, shelf life listed as expiration date on bottle, low cost.

2.3.1.2. Reagent shelf life

The Molecular Probes Griess reagent, chosen as the highest rated test kit, was tested for application in prolonged unattended deployments with a shelf life test. Previous work reports that APHA Griess reagent pre-mixed and stored in the dark is stable about 5 days (Hansen and Koroleff, 1999) and a month when refrigerated (Eaton et al., 2005). A shelf life experiment was conducted to test the effectiveness of the Molecular Probes' Griess reagent over time by comparing the absorbance readings of nitrite standards with shelved, pre-mixed reagent (stored at room temperature in the dark) against the absorbance readings of nitrite standards mixed with freshly-made reagent.

The results suggest a 3-4 week approximate shelf life before the mixed reagent loses appreciable sensitivity (Figure 8a). After week 5, the calibration curve slope (0-10 μM NO_2^-) of the shelved Griess reagent fell outside the range of the mean slope value of the fresh reagent (>99% confidence). Beginning at week 4, the signal value of the standards mixed with shelved reagent fell by over 5% (Figure 8b) from the signal produced with the fresh reagents, with the low standards (0.25, 0.5, 1.0 μM) dropping at a higher percentage than the high standards (5,10, 25 μM) (Figure B.3 in Appendix B). The shelved reagent showed good linearity through 9 weeks with an average R^2 of 0.9993 (Figure 8b).



a.

Weeks Shelved	Average Signal Difference (%)	Linearity (R^2)
1	1.1	0.9995
2	1.3	0.9996
3	1.4	0.9986
4	6.6	0.9988
5	15.7	0.9995
7	7.9	0.9997
8	14	0.9988
9	20.8	0.9997

b.

Figure 8. Shelf life testing of Molecular Probes Griess reagent. a. Plot of weekly comparisons of calibration curve slopes using shelved and fresh reagents. **b.** Tabulated results.

2.3.2. Instrument performance I

2.3.2.1. Detection system

Coordinating detector spectral response with emitter peak wavelength has been reported to increase analytical sensitivity (Sieben et al., 2010; Ahn et al., 2015). This effect proved to offer greater sensitivity in the prototype sensor by combining the green LED with green optical filter on the TAOS color sensor. At 540 nm, the spectral response of the TAOS color sensor was highest in the clear (unfiltered) channel, followed by the green, blue, and red channels. The green channel proved to offer the most distinction between a blank reading and a spiked reading, with a difference of 25 standard deviations between the average readings of the blank and the sample (n = 12), followed by the clear channel with over 6 standard deviations, the blue channel with 4 standard deviations, and the red channel with less than 1 standard deviation difference (Figure B.4 in Appendix B). Systematic errors outside the signal deviation threshold were attributed to air bubble formation in the optical pathway, one of the most common and significant interferences in wet chemistry fluidics (Worsfold et al., 2013). Air bubbles decreased the light intensity reaching the light sensor, lowering the signal beyond the range of error attributed to random noise. Such a signal was used as both a quality control parameter and as a control indicator by signifying the passing and approaching of sample plugs.

2.3.2.2. Fluidics

Pump calibration proved to be linear using the millisecond counter ($R^2 = 0.999$) over a range of 40 to 1,000 milliseconds (Figure B.6b in Appendix B). The pump head rotations tracked using magnets and the Hall-effect sensor were used to calibrate solenoid actuation and allowed for approximately 30 µL of reagent to be injected into the near center of a passing

sample plug of 1 mL volume during the automated procedure. By synchronizing sampling and injection cycles with the pulsation timing and duration of pump operation, precise volumes of reagent and sample were achieved (+/- approximately 2 µL, Table B.2 in Appendix B) at a maximum flow rate of approximately 35 µL/sec, or 2.1 mL/min. After 20 minutes of mixing and incubation time, the light transmission of the reaction was stable and had reached absorbance values at 99% of the transmission at 30 minutes, revealing satisfactory mixing levels for the time frame (Figure B.7a, b in Appendix B). The fluidics of the system enabled mixing and consistent flow with a relatively simple approach that was based on rFIA, whose characteristics include a laminar flow pattern, merged mixing zones, and direct injection of reagent into the sample (Zagatto et al., 2012). Additionally, automation of fluidic handling was made possible through the multicommutation of compact and lightweight solenoid valves (8 mm width), an approach proven to aid in instrument miniaturization, portability, sampling throughput, measurement selectivity and repeatability, and decreased reagent consumption (Rocha and Reis, 2000; Feres and Reis 2005; Ródenas-Torralba et al., 2006; Melchert et al., 2007; Morales-Rubio et al 2009).

2.3.3. Instrument performance II

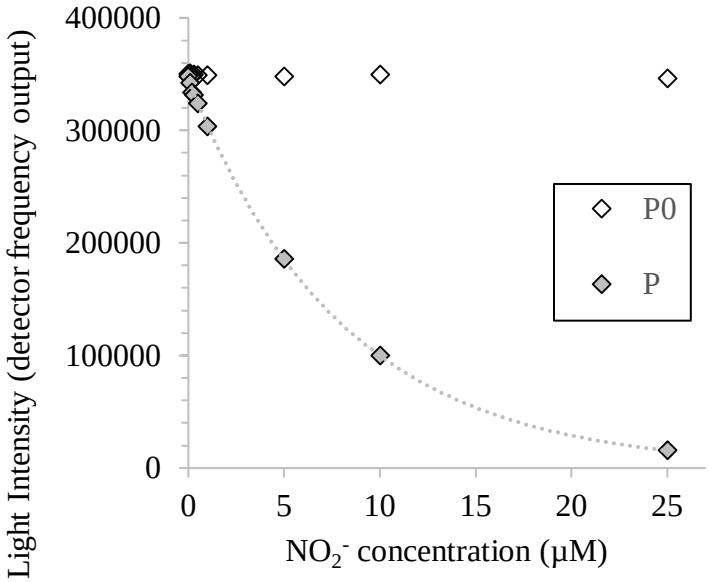
2.3.3.1. Calibration

The detection system was initially tested using FD&C red food dye no. 3 as proxy for the Griess reagent and demonstrated the ability of the system to operate as a chemical colorimeter with a high degree of linearity (R^2 = 0.997) and precision (relative standard deviation (RSD) = 0.1%) (Figure B.8 in Appendix B) at concentrations comparable to previous studies (Sieben et al., 2010; Bui and Hauser, 2015). After the food dye test, standard curves were constructed using nitrite standards ranging from 0.25 - 25 µM for both 5 cm and 2 cm flow-cells. Light

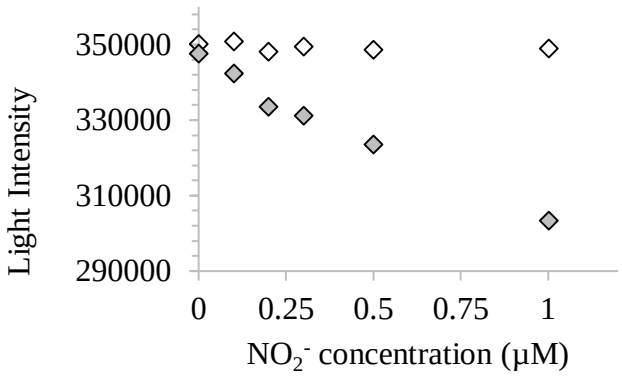
transmission among the standard solutions exhibited an exponential decrease, following the Beer-Lambert law (Figure 9a). Transmission was converted to absorbance using equation 2. Calibration using nitrite standards demonstrated the method's high degree of linearity and dynamic range without dilution up to 25 µM (Figure 9c).

Accurate detection depended on the establishment of a proper reading of ambient sample ($\boldsymbol{P_0}$) water to compare sample absorbance readings against (Figure 7). A working average RSD of 0.1% over 121 separate blanks in 9 separate calibration tests established a consistent baseline towards a precise absorbance measurement. Carryover between standards was effectively eliminated by the module's rinse cycle, and the average recovery to a blank signal was greater than 99% (n = 95).

a.



b.



c.

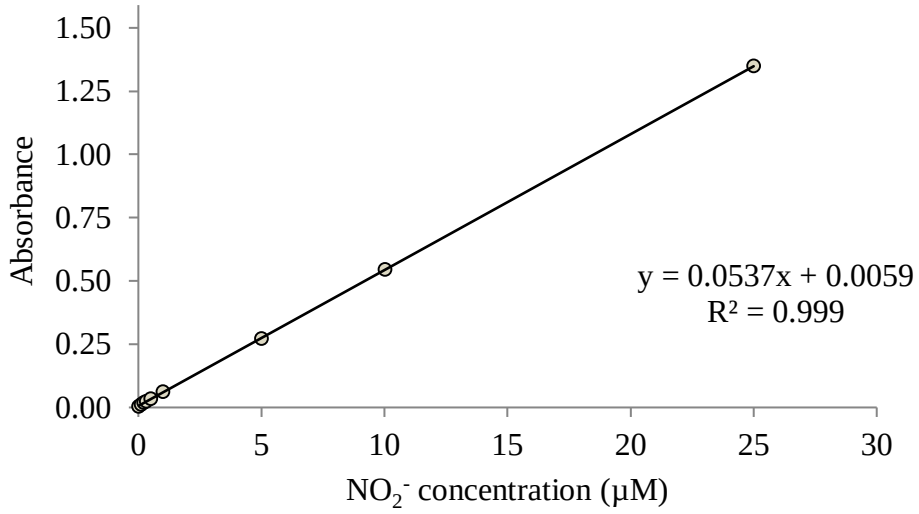
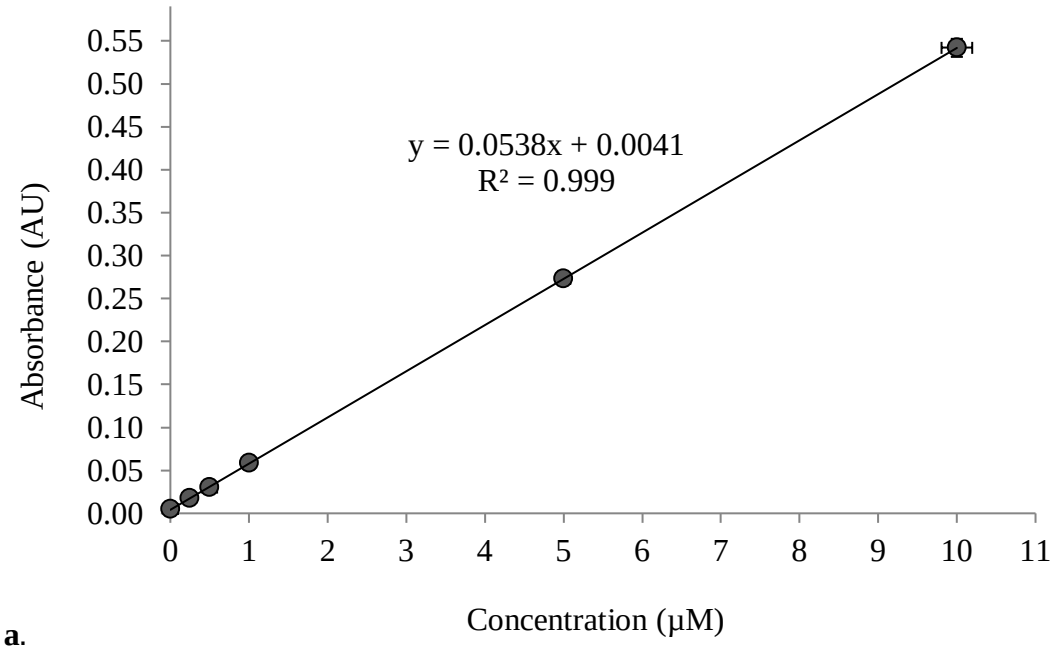


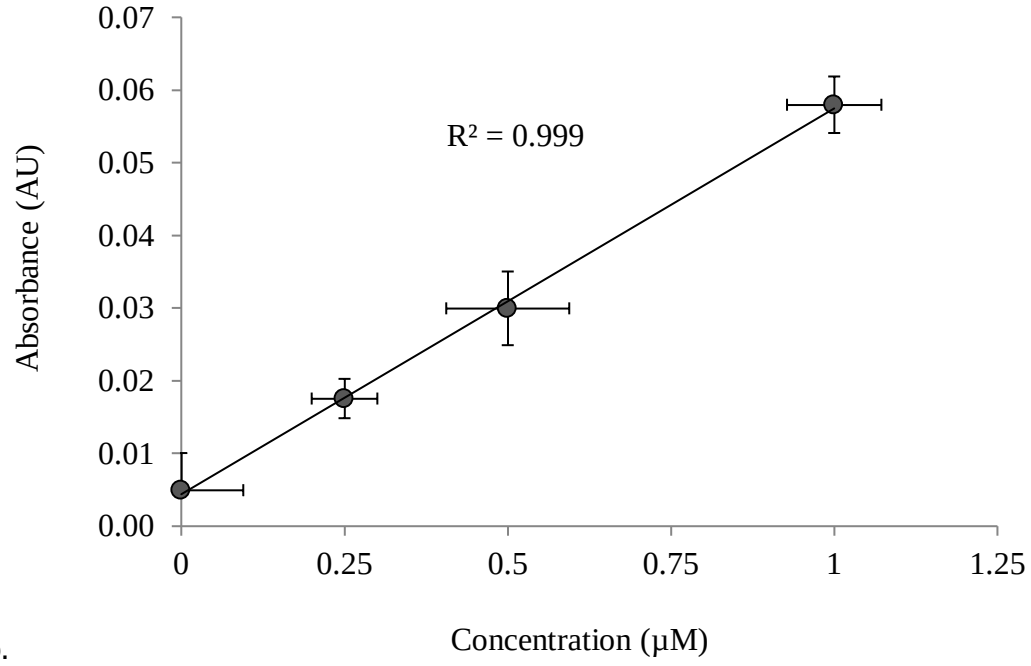
Figure 9. Calibration plot using 20 mm flow-cell and Molecular Probes reagent. a. Light intensity readings of ambient sample ($\boldsymbol{P_0}$) and analyzed sample ($\boldsymbol{P}$). **b.** Expanded view of low range standards transmission plot. **c.** Resulting absorbance

2.3.3.2. Limit of detection

The sensitivity and repeatability of the instrument was determined under a fully automated analysis routine that used nitrite standards ranging from 0.25 – 25 μM and a 2 cm pathlength PEEK flow-cell. A total of 5 replicates for each standard were performed, with each standard manually switched out before introduction of the next standard solution. Instrument sensitivity is limited by both the slope of the analytical curve and the reproducibility of the signal measurement (Skogerboe and Grant, 1970). The precision of prototype nitrite sensor depended on its ability to consistently account for air bubbles or other optical interferences, inject precise volumes, mix and manipulate the sample plug, and finally detect light transmission. The average inter-assay reproducibility produced a RSD of 1.4%, and an average intra-assay repeatability of 2.5% (n=30). The precision resulted in a minimum detectable signal of 0.01 absorbance units (n=30), calculated as 3 times the standard deviation of the blank added to the mean blank signal. When related to the calibration curve equation, the minimum detectable signal yielded a limit of detection of 0.18 μM (Figure 10a, b).



a.



b.

Figure 10. Instrument precision of prototype nitrite analyzer. a. 5x replicate standard curve (error bars as 3x standard deviation) **b.** Expanded view of low range standards

2.3.3.3. Method robustness

To test the ruggedness of the automated analytical process using the prototype system, over 100 absorbance measurements of nitrite standards were used to construct a calibration curve with confidence and prediction intervals (Figure 11). The measurements (n=105) were taken over 10 separate tests on different days, with standards (n=10, 0-25 µM) from various serial dilutions and stocks, with multiple Molecular Probes Griess reagents from varying storage conditions (e.g., stored premixed and at room temperature for up to 2 weeks, or stored separately at 4°C), and under small procedural differences with slight variations in instrument software and physical configuration (i.e., replacement of tubing, valves).

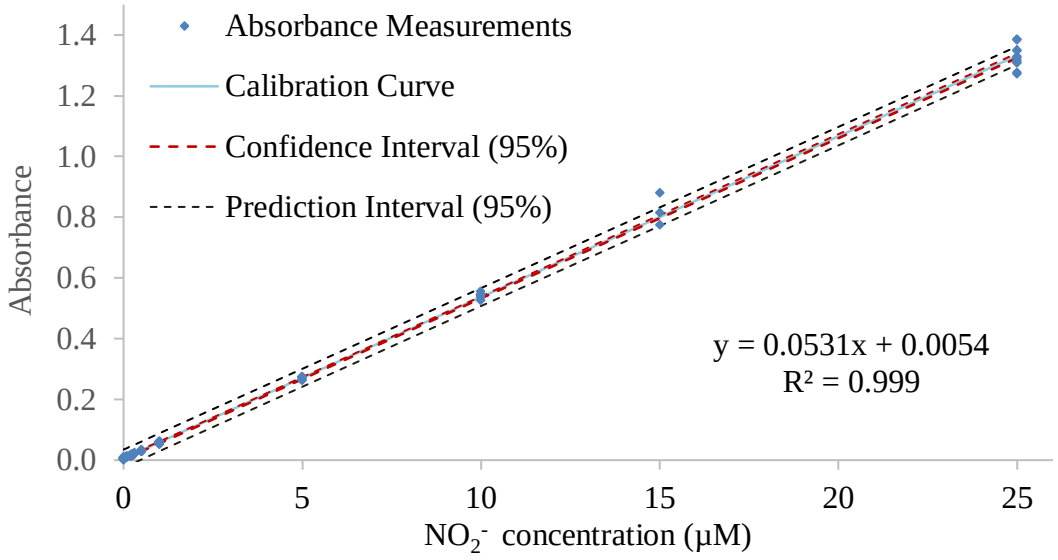


Figure 11. Global calibration curve. Plot of calibration curve made of absorbance measurements (n=105) using prototype nitrite sensor (2 cm flow-cell).

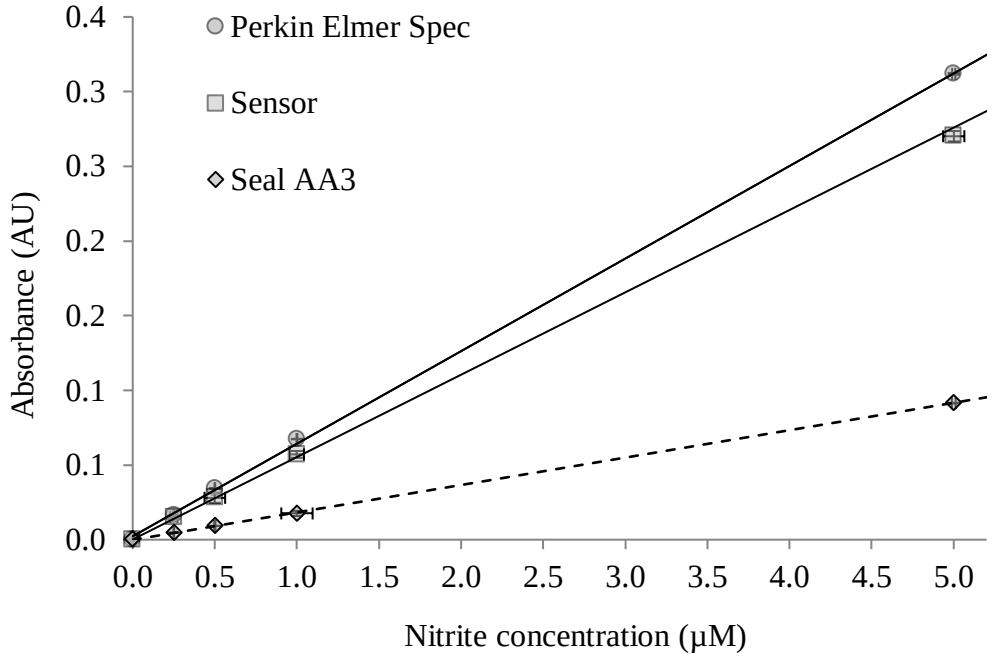
The nitrite standards' signals were unaffected by these changes and fell within the 95% prediction interval from 0-10 µM; at concentrations greater than 10 µM, the precision suffered (Figure 11). This result is to be expected as literature values report the linear range of the Griess assay to be near 10 µM with a 2 cm pathlength (Hansen and Koroleff, 1999, Eaton et al., 2005). The resulting calibration curve demonstrated the method's overall robustness and could be applied to calculate concentrations from absorbance measurements in prolonged and unattended operation.

2.3.3.4. Instrument comparison and validation

The module was compared to conventional methods and instrumentation by analyzing the same set of nitrite standards (0 – 10 µM) on a SEAL AA3 autoanalyzer and a Perkin Elmer Lambda 25 spectrophotometer. The Molecular Probes' manual method on the Perkin Elmer with a 2 cm cuvette was used as the reference method for comparison, and the prototype nitrite sensor produced a slope at 89% of the reference method's calibration curve slope (Figure 12a, b). The SEAL's sensitivity differed fundamentally from both the reference method and the prototype sensor because of a 1 cm cuvette pathlength that is used in the system; additionally, the Griess reagent was made according to the APHA standard method.

The prototype system also demonstrated a high degree of linearity and low level of variance comparable to the reference method and the autoanalyzer. The average inter-assay precision percentage for the nitrite sensor was an order of magnitude greater than the reference method (Figure 12b).

a.



b.

	Perkin Elmer Spec.	SEAL AA3	Prototype Nitrite Sensor
Slope	0.06190	0.02010	0.05510
Linearity (R^2)	0.99996	0.99998	0.99979
Residual Stdev. (AU)	0.00194	0.01094	0.00390
Slope Stdev.	0.00023	0.00131	0.00047
Avg. precision as signal RSD (n=5)	0.4%	2.3%	5.5%
Avg. precision as conc. (n=5)	±0.013 µM	±0.084 µM	±0.14 µM

Figure 12. Instrument comparison results. a. Plot of calibration curves (error bars as standard deviation, n=5). **b.** Tabulated results showing comparative performance of each instrument.

The accuracy of the instrument was validated against a Perkin Elmer Lambda 25 spectrophotometer (as the reference method) and the SEAL with recirculating aquaculture system (RAS) rearing tank water (Table 5). The samples were taken from a RAS whose biological filter is periodically backflushed into the tank, which causes a temporary increase in nitrite level. Three samples were tested – one before the backflush to represent typical operating conditions, one 45 minutes after, and one 165 minutes after. The post-backflush samples were

filtered through 0.2 µm pore glass fiber filters and diluted with type 1 water at a 1:100 ratio. The samples were analyzed on the nitrite sensor using a 5 cm flow-cell and quantified using a global calibration curve recorded with nitrite standards ranging from 0.1 to 2 µM from 5 calibration curve data sets. The results revealed an average absolute difference of 1.9 µM between the prototype nitrite sensor and the Perkin Elmer (reference method), a 3.3 µM average absolute difference between the SEAL and the Perkin Elmer, and a 2.5 µM average absolute difference between the nitrite sensor and the SEAL (Table 5).

Table 5. Instrument comparison. RAS tank water tested on 3 different systems.

	Perkin Elmer Spec.	SEAL AA3	Nitrite sensor
Sample	Conc.^ (µM)	Conc.^ (µM)	Conc.^ (µM)
RAS 45 min after backflush	33.44 ± 0.07	31.39 ± 1.98	29.78 ± 0.04
RAS 165 min after backflush	18.36 ± 0.07	14.05 ± 1.92	16.88 ± 0.04
RAS Pre-backflush	0.43 ± 0.07	1.93 ± 1.91	0.92 ± 0.04

2.3.4. Conclusion

The analytical process of the automated nitrite sensor has demonstrated the ability to detect and determine nitrite levels below 0.2 µM, with a linear range up to 25 µM and within 10% accuracy at micro-molar concentrations. The method was also relatively simple and low-cost relative to the reference methods, and reduced the complexity and components required of a simultaneous double-beam method. Additionally, the detection of light transmission of the blank served to correct for any background turbidity or other potentially interfering matrix effects.

The modular nature of the sensor package allowed for switching out of flow-cells and reagents for desired sensitivity and prolonged reagent shelf life, and featured a design with potential for other colorimetric assays to be carried out within the same manifold setup. Initial reagent results suggest that aquaculture technicians can mix the Molecular Probes' reagents at a 1:1 ratio and deploy for over 2 weeks, with each sample requiring less than 50 µL. The nitrite standard in the Molecular Probes' test kit also offers the potential for further development of automated calibration check protocols. With the exception of the PVC chassis and optical adapters for the RGB detector and LED, all components are commercially available and can be made and operated with off-the-shelf components at less than US$1,000.

The Arduino microcontroller has provided capabilities for complete automation and control through a user-driven interface with programmable sampling intervals, volumes, and operation cycles. Furthermore, the module can be further enhanced by integrating additional components with the Arduino microcontroller. The prototype system approaches the analytical merit necessary for scientific analysis, is easily accessible for technicians, and has potential for use as a process control tool in industrial aquaculture systems.

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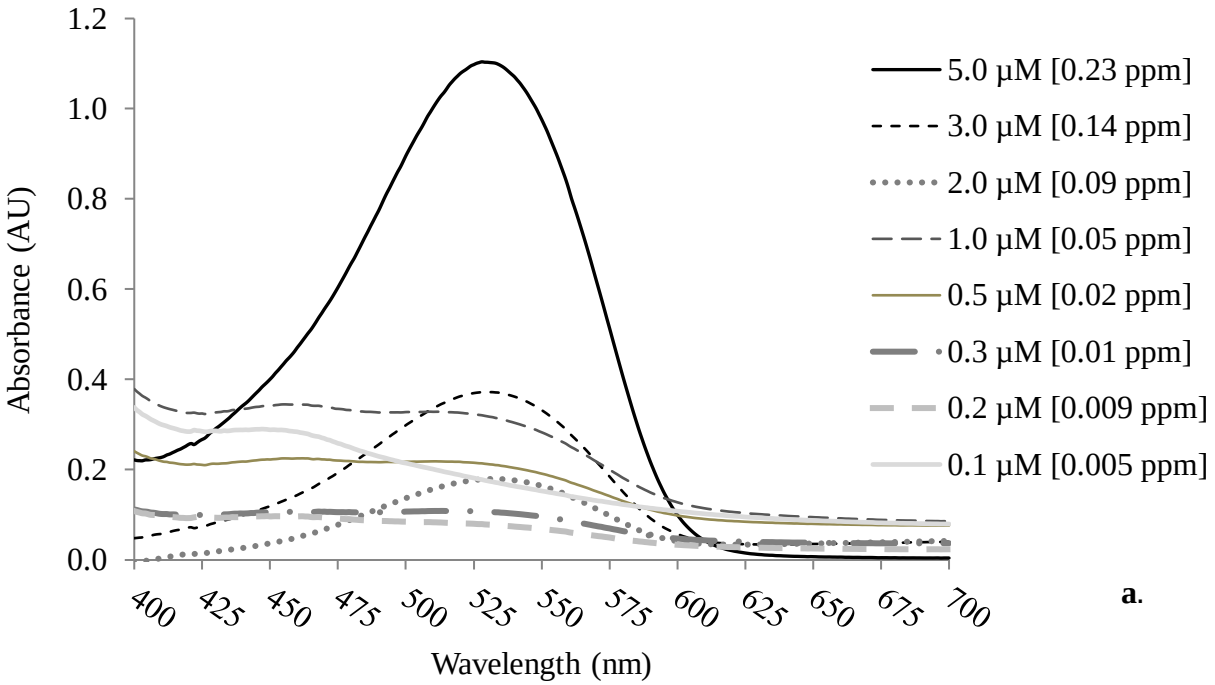
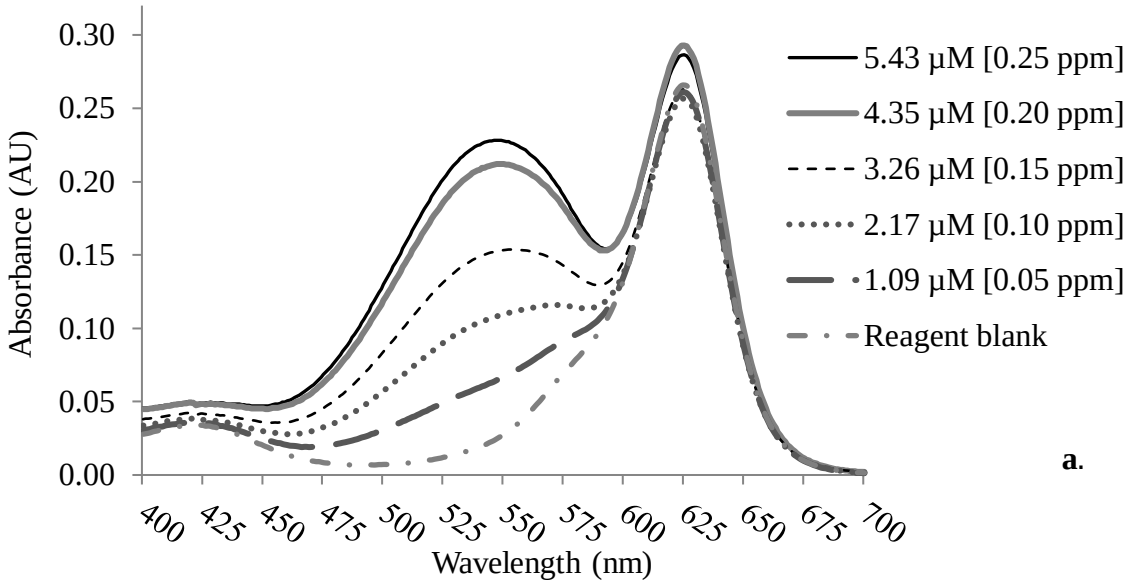
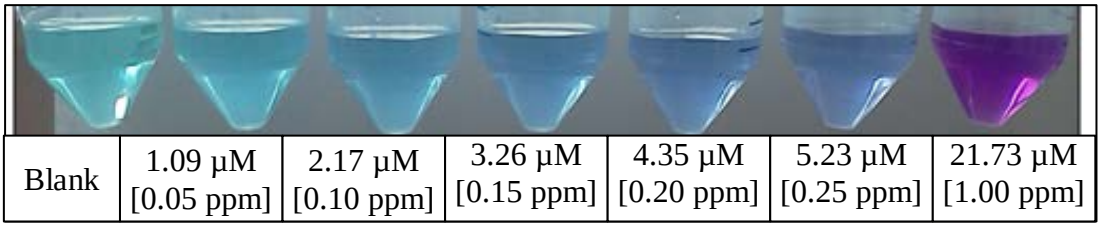
2.5. Appendix B: Supporting figures and tables



0.10 µM [0.005 ppm]	0.20 µM [0.009 ppm]	0.30 µM [0.01 ppm]	0.50 µM [0.02 ppm]	1.0 µM [0.05 ppm]	2.0 µM [0.09 ppm]	3.0 µM [0.14 ppm]	5.0 µM [0.23 ppm]

Figure B.1a. ELOS reagent + NO_2^- absorbance spectrum. Blank-corrected and tested on a Perkin Elmer Lambda 25 spectrophotomer in a 10 cm quartz cuvette. **b.** ELOS color card and standards.





Blank	1.09 µM [0.05 ppm]	2.17 µM [0.10 ppm]	3.26 µM [0.15 ppm]	4.35 µM [0.20 ppm]	5.23 µM [0.25 ppm]	21.73 µM [1.00 ppm]

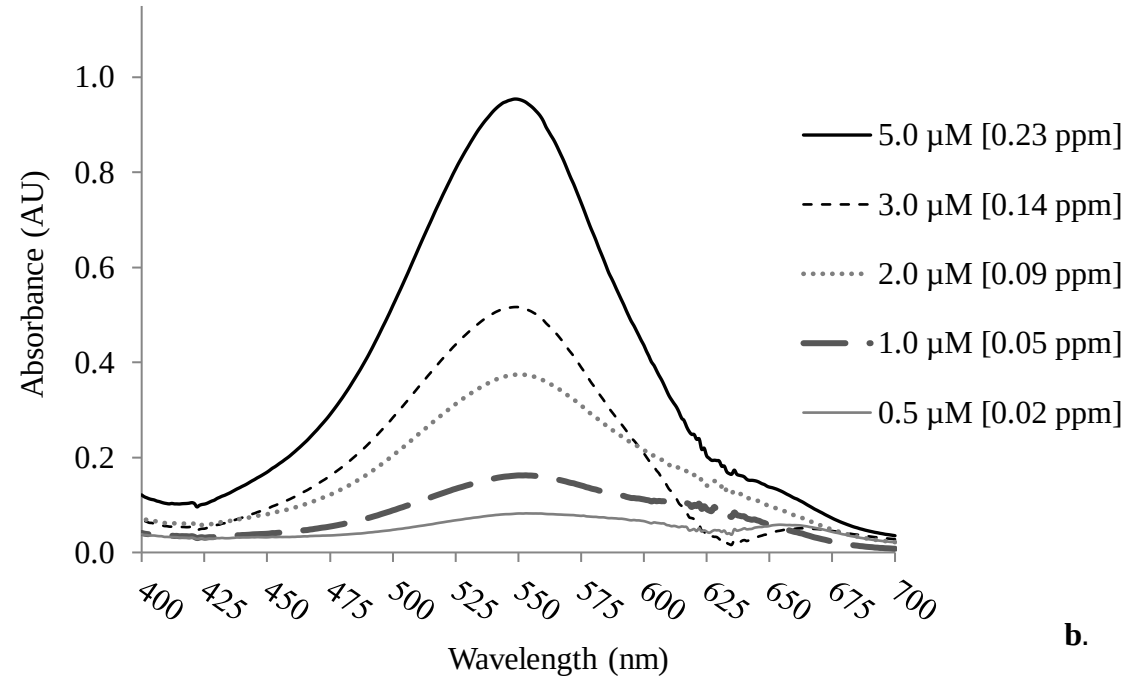


Figure B.2a. API reagent + NO_2^- absorbance spectrum tested on a Perkin Elmer Lambda 25 spectrophotomer in a 10 cm quartz cuvette. **b.** API reagent + NO_2^- absorbance spectrum blank-corrected.

Protocol	Components	List Price USD	Volume Supplied	Volume Required / Sample	Price / Sample
AWWA 4500-NO_2^- B	85% phosphoric acid	$198.08	1 L	200 µL	$0.07
	sulfanilamide	$35.88	100 g	20 mg	
	N-(1-naphthyl)-ethylenediamine dihydrochloride	$132.33	25 g	2 mg	
API	API nitrite reagent solution	$9.00	37 mL	150 µL	$0.05
Molecular Probes	Molecular Probes nitrite kit reagent A	$128.00	25 mL	50 µL / 10 µL*	$0.26 / 0.05*
	Molecular Probes nitrite kit reagent B		25 mL	50 µL / 10 µL*	
ELOS	ELOS nitrite reagent solution	$21.99	20 mL	350 µL	$0.40
Hach	Hach nitrite reagent powder	$37.39	1 pillow / test	100 pillows	$0.37

* = microplate assay

Table B.1. Reagent comparison – cost per assay.

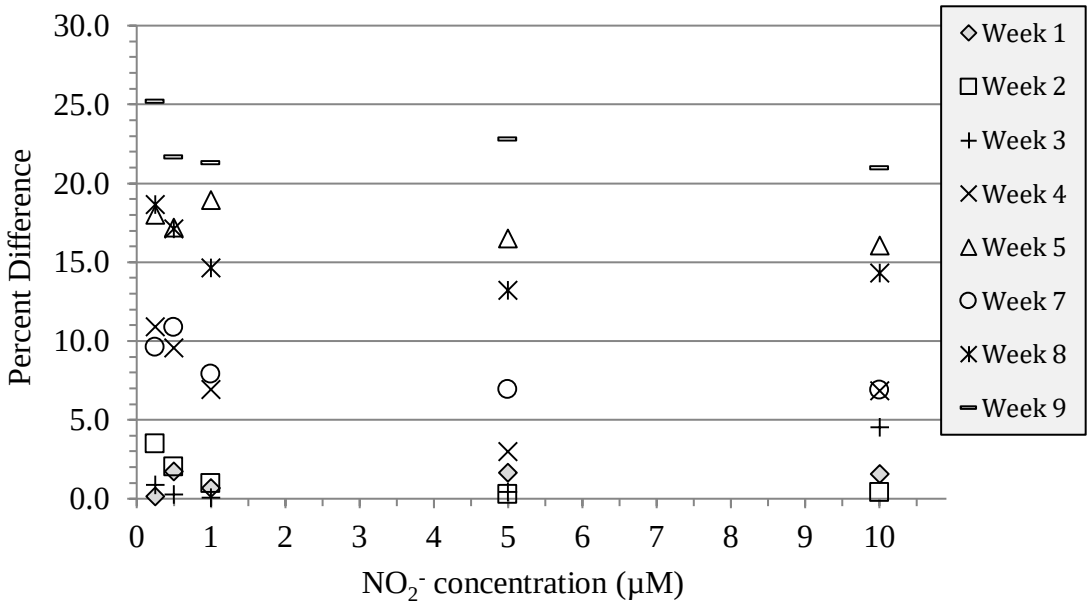


Figure B.3. Molecular Probes shelf life reagent test results: Average percentage difference in signal (absorbance) between fresh and shelved reagents.

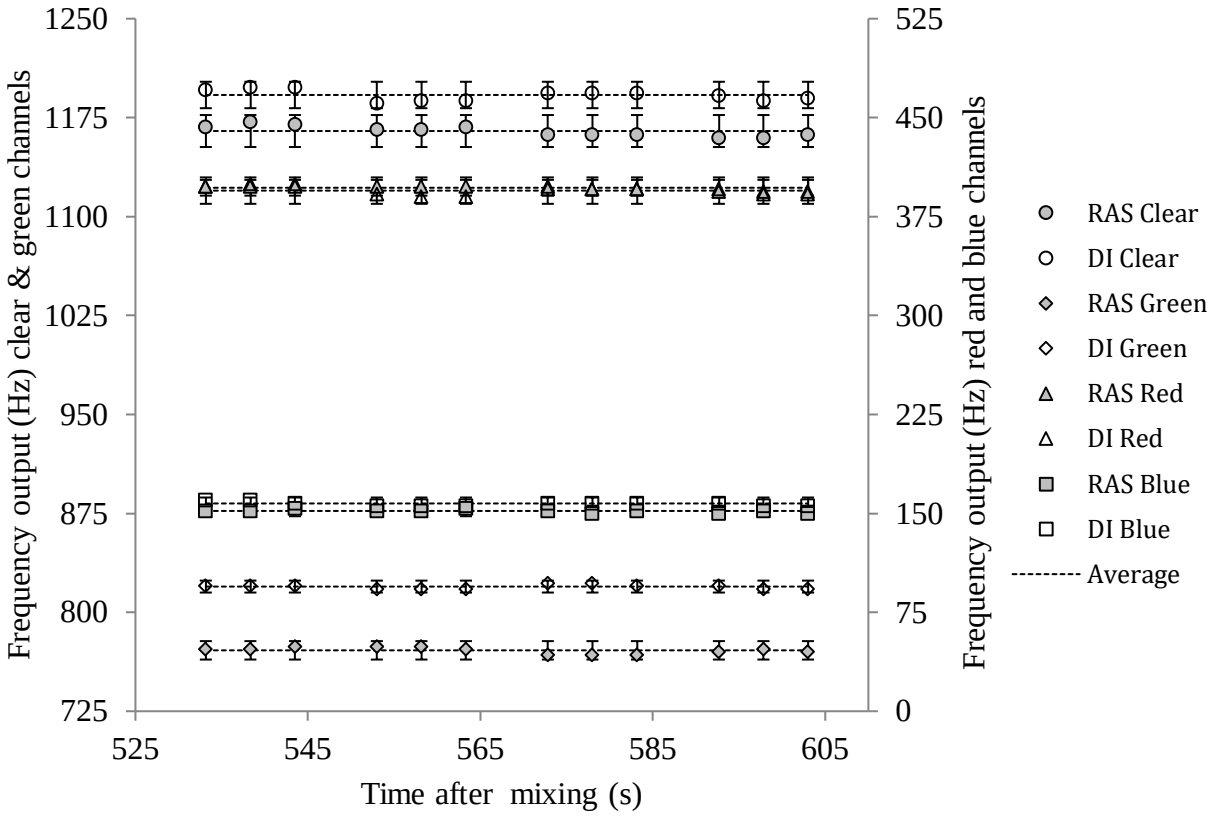


Figure B.4. Prototype nitrite analyzer signal output of API reagent and recirculating aquaculture system (RAS) tank water across 3 color filter channels (red, green, and blue), and clear (unfiltered). Error bars as 3x standard deviation, n = 40.

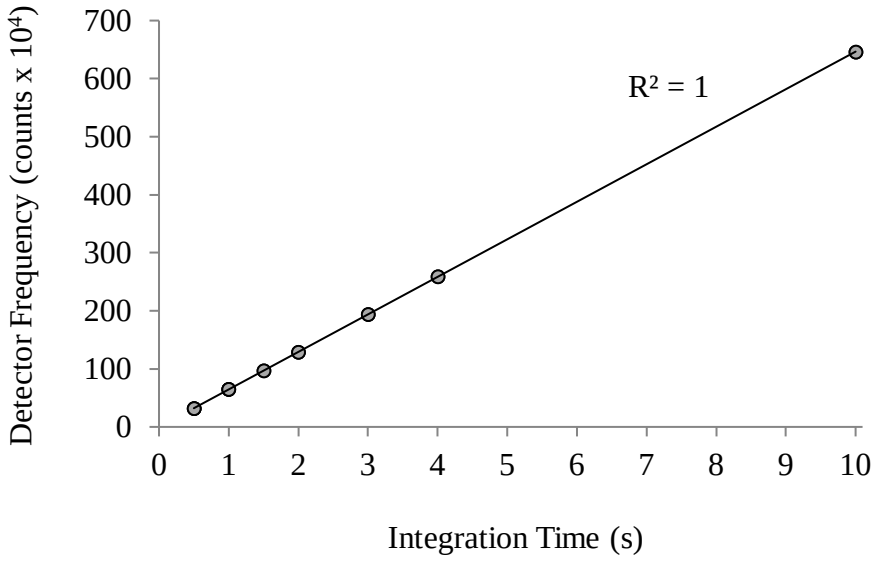


Figure B.5. Prototype nitrite analyzer light readings on a sample plug over varying integration times, beginning with 500 milliseconds.

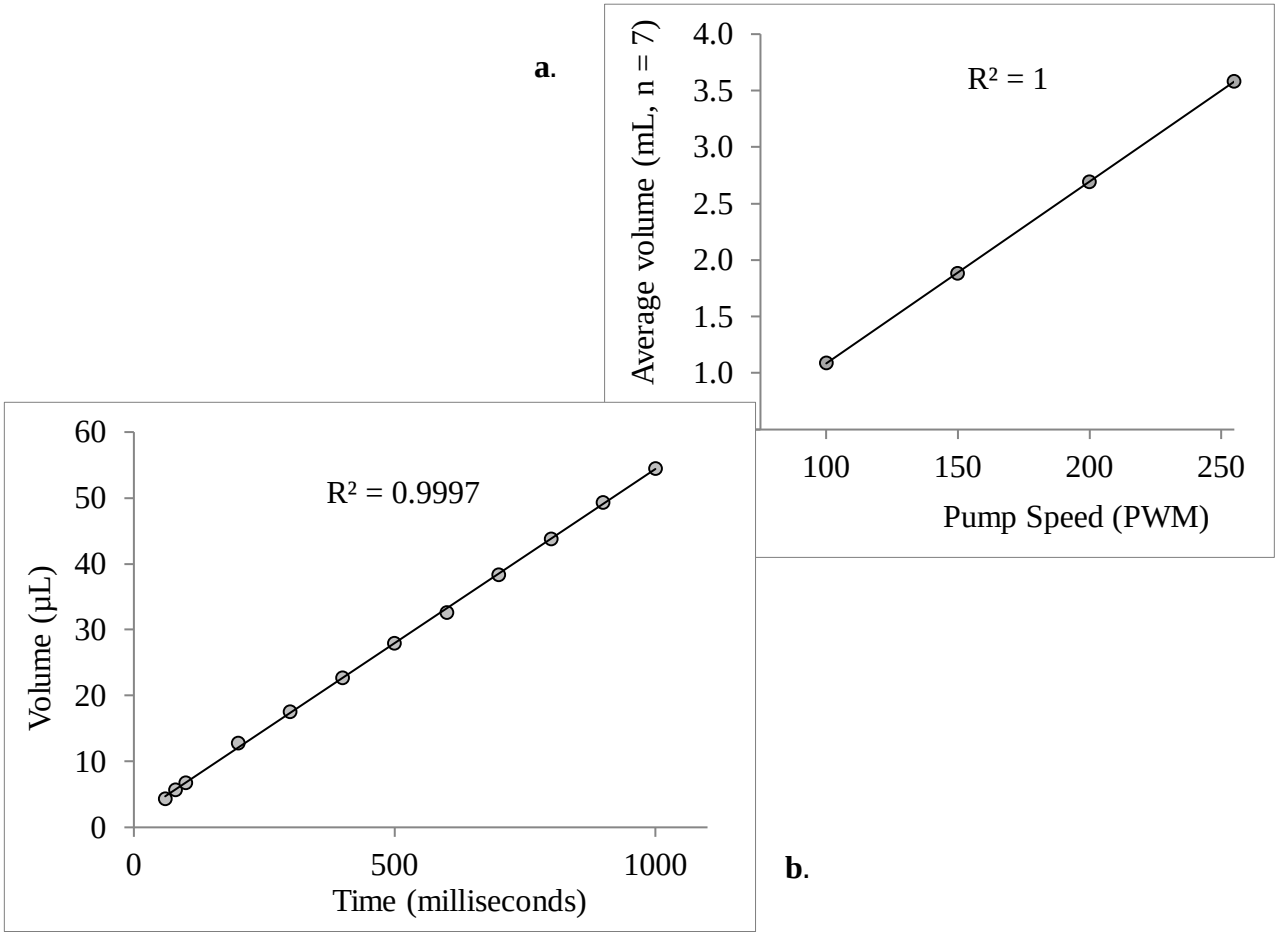
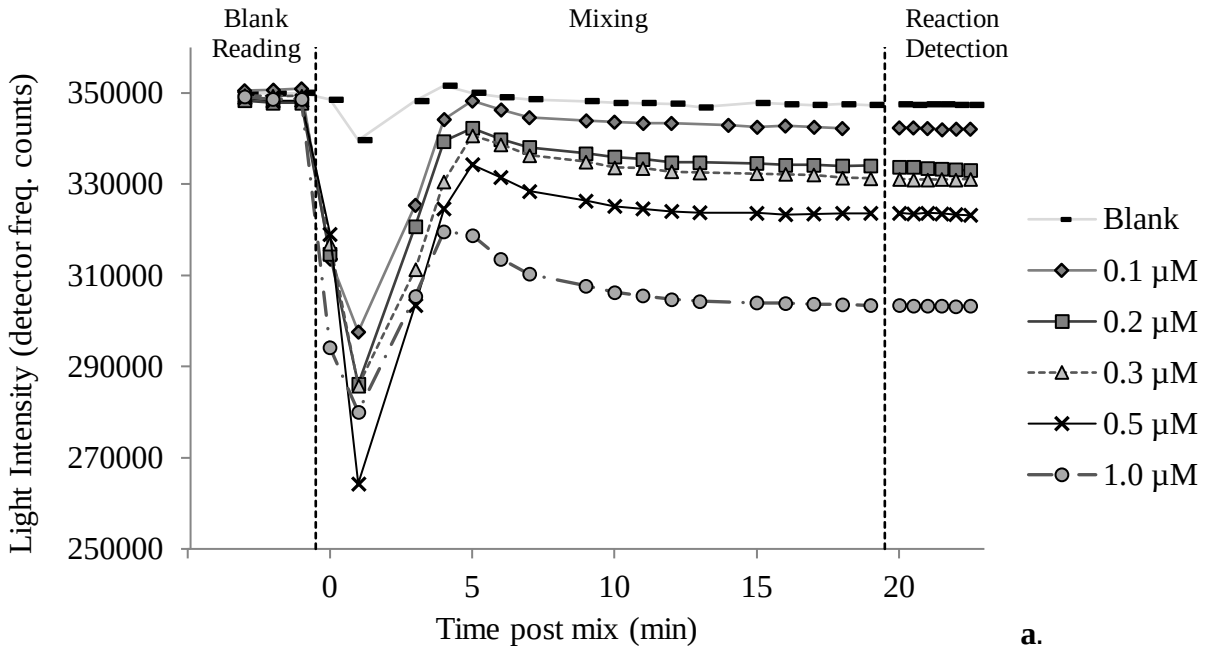


Figure B.6a. Pump calibration at varying speeds over 60 seconds using pulse width modulation and **b.** Pump calibration at constant speed and varying time.

Table B.2. Example results of pump calibration test using Hall Effect sensor to track 1 pump revolution and measurement of corresponding mass difference of Type 1 water drawn.

Before (mg)	After (mg)	Difference (mg)
32.5093	32.4745	0.0348
32.4747	32.4401	0.0346
32.4400	32.4051	0.0349
32.4053	32.3715	0.0338
32.3716	32.3366	0.0350
32.3367	32.3019	0.0348
32.3016	32.2652	0.0364
32.2652	32.2320	0.0332
32.2320	32.1932	0.0388
32.1932	32.1543	0.0389
32.1545	32.1185	0.0360



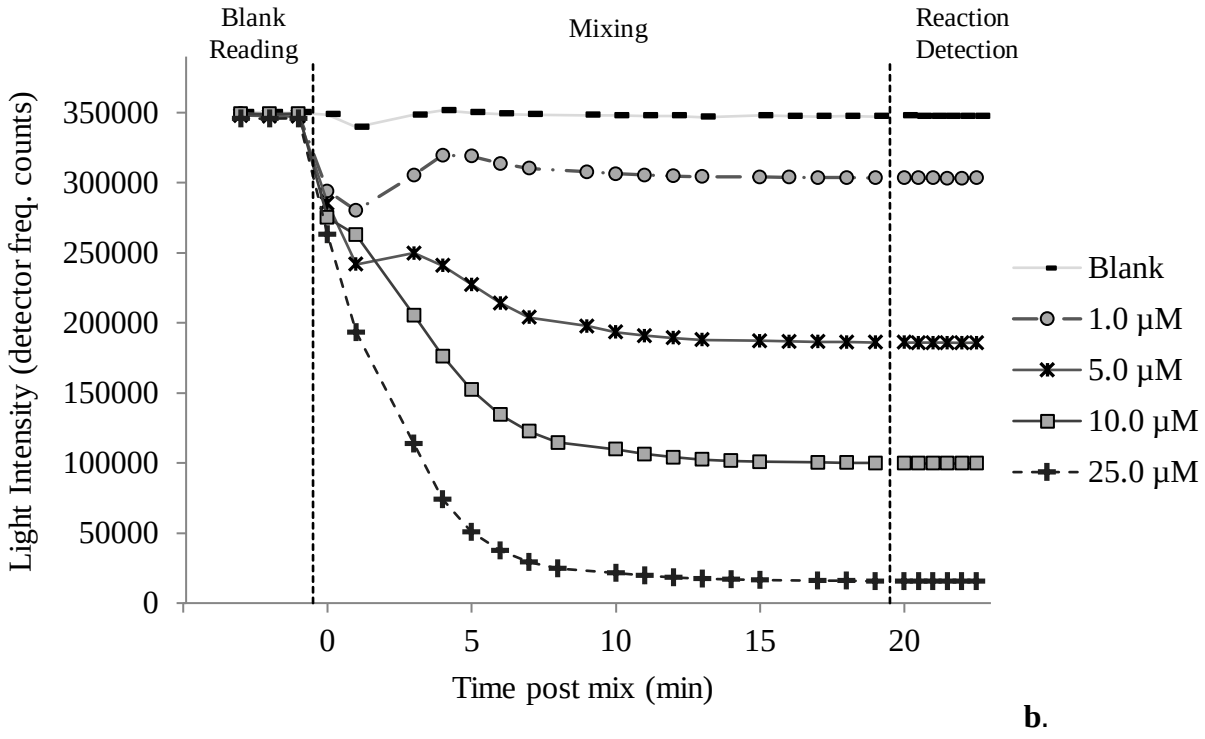


Figure B.7a. Reaction kinetics of Molecular Probes nitrite reagent on prototype nitrite analyzer with 20 mm flow-cell over low range standards and **b.** over high range standards.

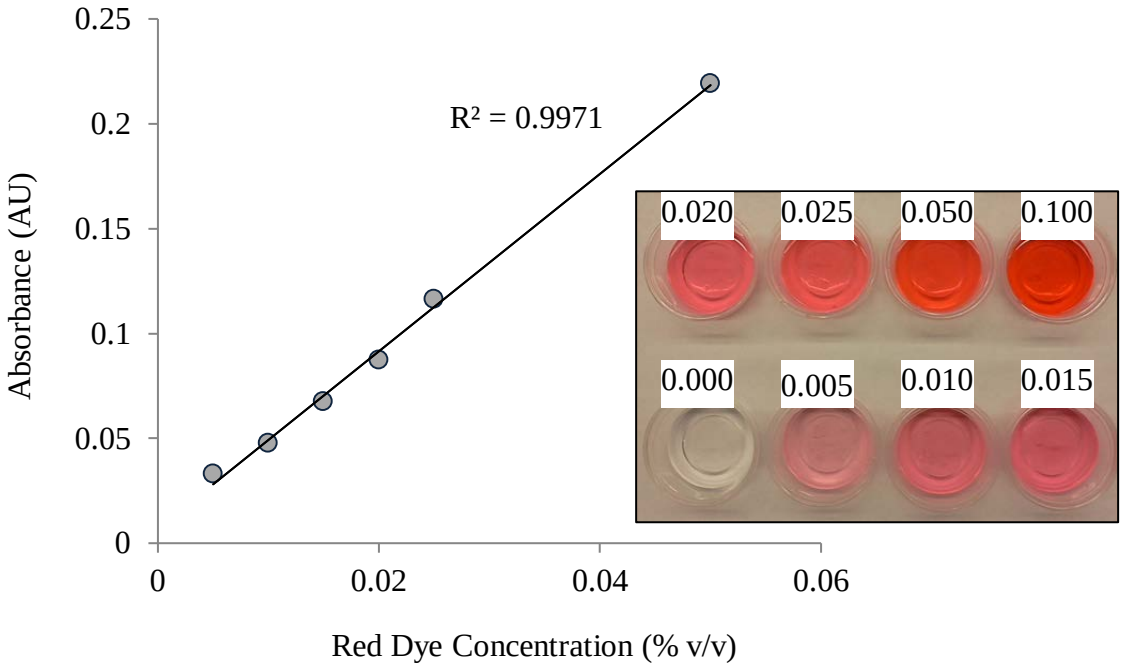


Figure B.8. Result of red food dye calibration curve on prototype nitrite analyzer. Standards manually pumped through 20 mm flow-cell with syringe.

2.6. Appendix C: Data tables

Table C.1. Reagent comparison

Standard (µM)	Mean Abs.	+/- Std. Dev.	Ɛ (M^{-1} cm^{-1})	λ (nm)	b (cm)
Griess reagent (Standard Method – manual in lab.)					
0.1	0.0407	0.0004	40733	543	10
0.2	0.0808	0.0003	40383	543	10
0.3	0.1206	0.0005	40211	543	10
0.5	0.2015	0.0003	40307	543	10
1	0.4007	0.0002	40070	543	10
2	0.8059	0.0005	40297	543	10
3	1.2017	0.0007	40058	543	10
5	1.9825	0.0027	39650	543	10
Molecular Probes nitrite reagent					
0.25	0.0447	0.0004	35787	548	5
0.5	0.0846	0.0001	33853	548	5
1	0.1717	0.0002	34340	548	5
5	0.8579	0.0025	34317	548	5
10	1.6338	0.0011	32677	548	5
25	3.1486	0.0010	25189	548	5
API nitrite reagent					
0.1	0.01630	0.00221	16300	550	10
0.2	0.02473	0.00145	12367	550	10
0.3	0.04473	0.00327	14911	550	10
0.5	0.08197	0.00159	16393	550	10
1	0.16217	0.00055	16217	550	10
2	0.37370	0.00151	18685	550	10
3	0.51607	0.00116	17202	550	10
5	0.95267	0.00087	19053	550	10
ELOS nitrite reagent					
0.1	0.35267	0.02898	NA	400	10
0.2	0.11263	0.00861	NA	400	10
0.3	0.11813	0.01273	NA	400	10
0.5	0.25140	0.01571	NA	400	10
1	0.39070	0.02002	NA	400	10
2	0.18477	0.00883	NA	530	10
3	0.37227	0.01128	NA	530	10
5	1.10113	0.00442	NA	530	10

Table C.2. Reagent shelf life test data

5 cm cuvette
548 nm

Zero week	Serial dilution from certified stock solution				
	Abs.				
Standard (µM)	Rep 1	2	3	4	5
Reagent Blank	0.0000	0.0000	0.0001	0.0000	0.0001
0.25	0.0468	0.0467	0.0468	0.0467	0.0468
0.5	0.0842	0.0842	0.0844	0.0843	0.0845
1	0.1745	0.1745	0.1744	0.1743	0.1744
5	0.8446	0.8449	0.8443	0.8437	0.8437
10	1.6447	1.6440	1.6428	1.6438	1.6440
25	3.1134	3.1265	3.1435	3.1434	3.1449

1 week	Serial dilution from 1mM working solution		
New reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0002	0.0002	0.0004
0.25	0.0436	0.0437	0.0435
0.5	0.0854	0.0843	0.0844
1	0.1720	0.1720	0.1719
5	0.8138	0.8138	0.8141
10	1.5506	1.5514	1.5519
25	3.0729	3.0657	3.0694

2 weeks	Serial dilution from 1mM working solution		
New reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0447	0.0444	0.0451
0.5	0.0845	0.0847	0.0847
1	0.1717	0.1719	0.1715
5	0.8571	0.8607	0.8560
10	1.6330	1.6334	1.6351
25	3.1483	3.1498	3.1478

1 week	Reagent 1		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0005	0.0000	0.0000
0.25	0.0436	0.0433	0.0434
0.5	0.0861	0.0860	0.0861
1	0.1706	0.1707	0.1707
5	0.7999	0.8003	0.8003
10	1.5266	1.5264	1.5272
25	3.0309	3.0461	3.0443

2 weeks	Reagent 1		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0430	0.0430	0.0435
0.5	0.0828	0.0828	0.0831
1	0.1700	0.1700	0.1700
5	0.8558	0.8553	0.8551
10	1.6400	1.6403	1.6405
25	3.1662	3.1622	3.1642

3 weeks	Serial dilution from 1mM working solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0002
0.25	0.0418	0.0420	0.0420
0.5	0.0821	0.0809	0.0821
1	0.1657	0.1656	0.1654
5	0.8209	0.8208	0.8213
10	1.6013	1.6002	1.6000
25	3.2633	3.2657	3.2666
3 weeks	Reagent 1		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0004	0.0002
0.25	0.0429	0.0424	0.0420
0.5	0.0816	0.0816	0.0816
1	0.1661	0.1654	0.1659
5	0.8250	0.8246	0.8246
10	1.5281	1.5281	1.5278
25	3.1924	3.1942	3.1952

4 weeks	Serial dilution from certified stock solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0434	0.0432	0.0433
0.5	0.0872	0.0870	0.0872
1	0.1732	0.1732	0.1732
5	0.8094	0.8097	0.8095
10	1.5712	1.5704	1.5715
25	3.0659	3.0856	3.0774
4 weeks	Reagent 1		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0010	0.0000	0.0010
0.25	0.0389	0.0384	0.0384
0.5	0.0788	0.0788	0.0788
1	0.1612	0.1611	0.1612
5	0.7855	0.7854	0.7847
10	1.4628	1.4631	1.4637
25	3.0059	3.0088	3.0157

5 weeks	Serial dilution from 1mM working solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0438	0.0438	0.0440
0.5	0.0874	0.0875	0.0876
1	0.1785	0.1793	0.1790
5	0.8497	0.8499	0.8499
10	1.6112	1.6118	1.6116
25	3.1108	3.1437	3.1540
5 weeks	Reagent 1		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0360	0.0359	0.0360
0.5	0.0723	0.0725	0.0725
1	0.1451	0.1448	0.1451
5	0.7090	0.7098	0.7096
10	1.3524	1.3522	1.3523
25	2.8877	2.9010	2.9097

7 weeks	Serial dilution from 1mM working solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0440	0.0440	0.0440
0.5	0.0842	0.0842	0.0842
1	0.1641	0.1641	0.1640
5	0.7845	0.7850	0.7855
10	1.5088	1.5091	1.5101
25	3.2141	3.2091	3.2275
7 weeks	Reagent 2		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0396	0.0398	0.0399
0.5	0.0750	0.0751	0.0750
1	0.1513	0.1513	0.1507
5	0.7313	0.7307	0.7300
10	1.4050	1.4058	1.4059
25	3.0450	3.0499	3.0513

8 weeks	Serial dilution from 1mM working solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0475	0.0475	0.0476
0.5	0.0926	0.0921	0.0922
1	0.1832	0.1831	0.1834
5	0.8779	0.8771	0.8770
10	1.6582	1.6581	1.6575
25	3.1259	3.1414	3.1394
8 weeks	Reagent 3		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0006	0.0003	0.0004
0.25	0.0390	0.0391	0.0392
0.5	0.0769	0.0770	0.0769
1	0.1568	0.1567	0.1569
5	0.7622	0.7617	0.7616
10	1.4223	1.4204	1.4206
25	2.9509	2.9572	2.9538

9 weeks	Serial dilution from 1mM working solution		
New Reagent Abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0002	0.0001	0.0001
0.25	0.0446	0.0447	0.0448
0.5	0.0882	0.0884	0.0883
1	0.1746	0.1787	0.1752
5	0.8481	0.8480	0.8480
10	1.5962	1.5955	1.5955
25	3.1011	3.1041	3.1002
9 weeks	Reagent 3		
Old reagent abs.			
Standard (µM)	Rep 1	2	3
Reagent Blank	0.0000	0.0000	0.0000
0.25	0.0332	0.0334	0.0334
0.5	0.0715	0.0678	0.0679
1	0.1384	0.1385	0.1387
5	0.6548	0.6545	0.6540
10	1.2613	1.2609	1.2605
25	2.7062	2.7095	2.7102

Table C.3. Instrument calibration: Nitrite sensor (2 cm flow-cell)

Reagent Blank				0.1			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	350102.0	347.9	15	1	350540.7	72.8	15
2	349877.4	58.6	15	2	350696.7	95.3	15
3	350053.4	35.1	15	3	350990.0	103.1	15
Analyze Blank...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	348564.7	11944.5	15	0	313683.4	25321.8	15
1	339658.0	4712.3	15	1	297622.0	9050.4	15
3	348269.4	1689.4	15	3	325432.0	2412.0	15
4	351630.7	1838.3	15	4	344252.0	1631.5	15
5	350041.4	893.6	15	5	348256.0	2029.6	15
6	349110.0	254.4	15	6	346341.4	1204.1	15
7	348657.4	168.7	15	7	344710.0	443.5	15
9	348172.7	171.0	15	9	344017.4	121.9	15
10	347877.4	159.7	15	10	343732.0	113.6	15
11	347803.4	118.1	15	11	343374.7	129.0	15
12	347622.7	198.3	15	12	343408.0	175.1	15
13	346845.4	201.5	15	14	343026.0	187.1	15
15	347876.0	153.0	15	15	342617.4	194.0	15
16	347562.7	166.6	15	16	342790.0	185.5	15
17	347352.0	160.2	15	17	342526.0	128.7	15
18	347562.7	108.8	15	18	342244.0	122.8	15
Read 1	347586.7	175.1	15	Read 1	342458.0	120.7	15
Read 2	347413.4	180.1	15	Read 2	342474.0	146.1	15
Read 3	347469.4	139.1	15	Read 3	342214.7	141.3	15
Read 4	347512.0	148.0	15	Read 4	341971.4	110.1	15
Read 5	347338.7	108.9	15	Read 5	342102.7	109.7	15
Read 6	347466.7	140.3	15	Read 6	342104.0	72.3	15

0.2				0.3			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	348322.7	282.1	15	1	349526.7	246.2	15
2	347855.4	121.7	15	2	349300.7	53.0	15
3	347869.4	80.8	15	3	349401.4	66.5	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	314622.7	29968.6	15	0	316977.4	40557.6	15
1	286137.4	9673.2	15	1	285792.0	10436.7	15
3	320733.4	1949.2	15	3	311249.4	4032.2	15
4	339432.0	1623.0	15	4	330558.7	1611.9	15
5	342307.4	2008.3	15	5	340710.7	2007.6	15
6	339824.7	1183.3	15	6	338746.0	1479.3	15
7	338104.0	571.2	15	7	336371.4	766.1	15
9	336781.4	172.1	15	9	334942.0	301.3	15
10	336026.0	119.9	15	10	333648.0	178.6	15
11	335466.7	98.5	15	11	333517.4	87.4	15
12	334832.0	96.8	15	12	332756.0	110.0	15
13	334830.0	95.4	15	13	332529.4	102.7	15
15	334565.4	70.7	15	15	332315.4	82.9	15
16	334212.7	62.4	15	16	332181.4	94.6	15
17	334209.4	65.5	15	17	332028.7	136.1	15
18	334038.7	69.9	15	18	331416.0	119.5	15
				19	331317.4	77.2	15
Read 1	333842.0	63.0	15	Read 1	331148.0	64.7	15
Read 2	333794.0	56.7	15	Read 2	331036.7	251.7	15
Read 3	333456.7	65.6	15	Read 3	331010.7	95.5	15
Read 4	333333.4	88.7	15	Read 4	331063.4	83.2	15
Read 5	333192.0	78.2	15	Read 5	331006.0	77.8	15
Read 6	333024.7	39.5	15	Read 6	331139.4	93.6	15

0.5				1.0			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	348895.4	391.4	15	1	349161.4	423.7	15
2	348260.0	61.3	15	2	348620.0	47.2	15
3	348323.4	76.1	15	3	348704.7	57.4	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	319068.0	38199.3	15	0	294144.0	35058.3	15
1	264342.0	9081.3	15	1	280077.4	9127.2	15
3	303494.7	3685.0	15	3	305476.7	2935.4	15
4	324602.0	1348.4	15	4	319656.0	2192.4	15
5	334306.7	2115.7	15	5	318817.4	2329.5	15
6	331470.7	1621.6	15	6	313563.4	1068.0	15
7	328456.7	696.7	15	7	310309.4	685.1	15
9	326394.0	240.7	15	9	307731.4	266.2	15
10	325207.4	175.6	15	10	306313.4	114.1	15
11	324630.0	65.7	15	11	305504.0	54.4	15
12	324062.0	113.6	15	12	304787.4	125.9	15
13	323751.4	84.8	15	13	304317.4	209.6	15
15	323678.7	129.8	15	15	304010.0	59.6	15
16	323362.7	110.2	15	16	303879.4	58.0	15
17	323504.7	107.4	15	17	303702.7	51.6	15
18	323664.7	184.0	15	18	303634.7	67.3	15
19	323619.4	137.2	15	19	303418.0	94.0	15
Read 1	323700.7	160.4	15	Read 1	303471.4	92.7	15
Read 2	323524.0	466.6	15	Read 2	303380.0	66.6	15
Read 3	323675.4	117.1	15	Read 3	303332.0	67.6	15
Read 4	323547.4	136.9	15	Read 4	303261.4	70.4	15
Read 5	323326.0	180.8	15	Read 5	303238.0	37.3	15
Read 6	323218.0	130.0	15	Read 6	303298.7	77.3	15

5.0				10.0			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	347998.0	408.4	15.0	1	349097.4	256.6	15
2	347454.0	50.7	15.0	2	349082.0	147.8	15
3	347346.7	38.1	15.0	3	349316.7	87.1	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	285265.4	33056.6	15	0	275126.7	37315.9	15
1	241840.7	14023.2	15	1	262813.4	21773.3	15
3	249705.3	7599.5	15	3	205480.0	11766.2	15
4	240856.7	5117.9	15	4	176183.3	8317.5	15
5	227441.3	3661.1	15	5	152176.0	5021.5	15
6	214091.3	2089.7	15	6	134317.3	2515.0	15
7	203932.0	1038.6	15	7	122468.7	1298.0	15
9	197638.7	532.6	15	8	114644.7	717.9	15
10	193352.0	272.2	15	10	109640.0	384.6	15
11	190839.3	133.0	15	11	106276.7	219.8	15
12	189102.0	79.6	15	12	103976.0	129.6	15
13	187910.7	78.7	15	13	102490.7	81.5	15
15	187203.3	24.4	15	14	101498.7	44.1	15
16	186770.7	37.5	15	15	100880.7	26.4	15
17	186445.3	58.2	15	17	100428.7	29.9	15
18	186182.7	35.1	15	18	100128.0	25.6	15
19	186049.3	50.4	15	19	99943.3	31.1	15
Read 1	185961.3	40.5	15.0	Read 1	99812.0	27.4	15
Read 2	185837.3	59.5	15.0	Read 2	99736.7	23.0	15
Read 3	185745.3	36.1	15.0	Read 3	99688.0	25.6	15
Read 4	185760.0	39.2	15.0	Read 4	99638.0	27.4	15
Read 5	185660.7	36.0	15.0	Read 5	99601.3	39.3	15
Read 6	185689.3	34.1	15.0	Read 6	99598.0	27.4	15

25.0

Blank Reading

Counter	Green Ave	Grn stDev	counts averaged
1	346366.7	520.5	15
1	345760.7	83.6	15
2	345752.7	70.0	15
3	345972.7	140.3	15

Analyze Standard...

Mix Counter	Green Ave	Grn stDev	counts averaged
0	263183.4	46562.6	15
1	193438.7	30474.9	15
3	113922.7	16407.1	15
4	73885.3	8387.9	15
5	50752.0	3649.8	15
6	37283.3	1592.8	15
7	29431.3	753.2	15
9	24586.7	402.9	15
10	21558.7	230.7	15
11	19595.3	143.7	15
12	18258.0	88.2	15
13	17368.0	59.6	15
15	16765.3	38.3	15
16	16349.3	24.1	15
17	16057.3	14.4	15
18	15848.0	11.7	15
19	15701.3	6.2	15

Read 1	15626.0	8.0
Read 2	15564.7	6.2
Read 3	15522.0	5.4
Read 4	15482.0	6.5
Read 5	15461.3	7.2
Read 6	15438.0	7.5

Table C.4. Instrument precision: Nitrite sensor (2 cm flow-cell)

Conc. (uM)	Replicate	Avg. Blank Read (n=3)	Stdev. Blank Read (n=3)	Avg. Blank Read (n=6)	Stdev. Blank Read (n=6)
0.00	1	353094.2	387.8	349154.1	569.4
0.00	2	352739.6	348.7	350783.6	133.5
0.00	3	354442.5	437.3	350766.9	169.4
0.00	4	355861.4	498.2	350892.4	108.2
0.00	5	356672.5	300.0	351082.9	131.9
0.25	1	354010.7	264.0	341165.9	192.9
0.25	2	355438.2	537.5	341412.2	203.4
0.25	3	355990.2	438.8	341249.6	199.7
0.25	4	355901.8	443.7	341536.6	263.7
0.25	5	356059.8	386.8	341590.7	340.9
0.50	1	352675.1	318.1	331126.6	269.4
0.50	2	355433.6	335.5	331382.1	406.0
0.50	3	355891.8	283.2	332423.6	571.4
0.50	4	356415.4	319.2	332328.1	444.1
0.50	5	357852.0	423.6	332416.7	141.2
1.00	1	361716.3	312.8	315403.1	425.0
1.00	2	359395.2	332.1	315115.2	678.5
1.00	3	360333.8	250.8	315120.3	641.0
1.00	4	360997.8	215.8	315302.2	544.4
1.00	5	355961.4	217.7	312705.7	386.0
5.00	1	358082.7	371.6	192240.3	1149.4
5.00	2	358662.3	305.3	191072.8	1453.8
5.00	3	357531.8	259.1	190635.6	2502.0
5.00	4	358156.2	320.2	190573.0	1189.9
5.00	5	359373.6	370.7	191749.2	822.9
10.00	1	362864.9	462.8	104808.2	1062.4
10.00	2	360566.9	310.2	103241.5	2212.7
10.00	3	361843.4	418.4	103302.3	2104.2
10.00	4	362032.7	403.4	103230.9	1410.6
10.00	5	361952.5	157.8	105148.8	649.5
25.00	1	359575.8	343.8	17592.1	860.6
25.00	2	361571.2	428.0	16968.2	458.0
25.00	3	360532.3	273.5	17718.2	1145.1
25.00	4	360383.2	300.0	17432.6	778.5
25.00	5	361024.2	576.6	17100.9	781.2

Table C.5. Instrument comparison: Perkin Elmer spectrophotometer (2 cm cuvette)

Sample (µM)	Absorbance (AU)	Sample (µM)	Absorbance (AU)
0.25	0.0162	RAS 45 min*	0.0238
0.25	0.0163	RAS 45 min*	0.0238
0.25	0.0163	RAS 45 min*	0.0238
0.25	0.0163	RAS 45 min*	0.0238
0.25	0.0164	RAS 45 min*	0.0238
0.5	0.0343	RAS 165 min**	0.1168
0.5	0.0343	RAS 165 min**	0.1166
0.5	0.0343	RAS 165 min**	0.1165
0.5	0.0343	RAS 165 min**	0.1167
0.5	0.0344	RAS 165 min**	0.1170
1	0.0673	Blank	0.0000
1	0.0674		
1	0.0673	RAS Pre	0.0296
1	0.0674	RAS Pre	0.0295
1	0.0674	RAS Pre	0.0296
Blank	0.0000	RAS Pre	0.0296
5	0.3117	RAS Pre	0.0294
5	0.3118		
5	0.3118	*1:100 dilution	
5	0.3122	**1:10 dilution	
5	0.3120		
10	0.6218		
10	0.6219		
10	0.6220		
10	0.6220		
10	0.6219		
25	1.3207		
25	1.3214		
25	1.3213		
25	1.3209		
25	1.3208		
Blank	0.0000		
Blank	0.0000		
Blank	0.0000		
Blank	0.0000		

Table C.6. Instrument comparison: SEAL AA3 (1 cm pathlength)

Sample (µM)	Absorbance (mAU)	Value (corrected)	Sample (µM)	Absorbance (mAU)	Value (corrected)
Baseline	0.00	0.027	10.00	183.72	9.167
Primer	495.95	24.912	10.00	183.40	9.149
Drift	494.80	24.831	10.00	184.22	9.188
25.00 std.	498.59	25.016	10.00	183.22	9.136
10.00 std.	199.07	9.988	1.00 std.	20.26	1.009
5.00 std.	98.13	4.937	25.00	455.83	22.73
1.00 std.	19.88	1.017	25.00	459.12	22.871
0.50 std.	9.52	0.5	25.00	457.17	22.768
0.25 std.	4.91	0.269	25.00	458.18	22.813
0.00 std.	-0.01	0.022	25.00	457.29	22.765
High	497.52	24.953	Blank	1.41	0.053
Low	5.55	0.276	RAS 4/3	37.94	1.893
Low	5.09	0.277	RAS 165 min**	28.84	1.437
Blank	0.27	0.034	RAS 45 min*	5.71	0.286
0.25	4.65	0.253	Diluted Sample	498.81	24.831
0.25	4.72	0.256	Final Base	0.00	0.027
0.25	4.70	0.255			
0.25	4.64	0.251			
0.25	4.71	0.254	*1:100 dilution		
0.50	9.12	0.474	**1:10 dilution		
0.50	9.28	0.481			
0.50	9.20	0.477			
0.50	9.17	0.475			
0.50	9.28	0.479			
1.00	18.39	0.934			
1.00	18.20	0.924			
1.00	18.35	0.93			
1.00	18.41	0.933			
1.00	18.44	0.934			
0.50 std.	9.88	0.505			
5.00	91.59	4.586			
5.00	91.28	4.565			
5.00	91.72	4.586			
5.00	91.29	4.563			
5.00	91.27	4.561			
0.00	183.28	9.151			

Table C.7. Instrument comparison: Nitrite sensor (2 cm flow-cell)

Reagent Blank				0.25			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	358644.7	562.6	15	1	360638.0	149.6	15
1	358231.4	104.7	15	2	360416.0	111.5	15
2	358548.7	96.6	15	3	360425.4	72.7	15
3	358887.4	84.4	15				
Analyze Blank...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	358557.4	27062.6	15	0	345310.7	28335.0	15
2	304376.0	8028.5	15	1	354219.4	8470.9	15
3	341765.4	1827.7	15	3	351177.4	2928.2	15
4	360223.4	1390.3	15	4	356615.4	1873.1	15
5	360154.0	1252.7	15	5	356022.0	1142.6	15
6	359594.7	705.5	15	6	354795.4	642.8	15
8	359281.4	307.9	15	8	353274.7	284.7	15
9	358986.7	135.9	15	9	352161.4	132.9	15
10	358739.4	147.5	15	10	351172.0	88.6	15
11	358616.7	147.9	15	11	350284.7	79.8	15
13	358203.4	94.1	15	12	349578.7	124.6	15
14	358245.4	111.1	15	14	348916.7	106.2	15
15	358152.0	117.1	15	15	348603.4	143.8	15
16	357885.4	125.9	15	16	348210.7	160.6	15
17	357972.0	89.7	15	17	347819.4	82.2	15
19	358019.4	130.9	15	19	347430.7	95.7	15
Read 1	357717.4	71.2	15	Read 1	347188.7	75.1	15
Read 2	357440.7	87.3	15	Read 2	347091.4	103.8	15
Read 3	357332.0	135.3	15	Read 3	346938.0	93.1	15
Read 4	357313.4	193.2	15	Read 4	346818.0	104.9	15
Read 5	356965.4	79.9	15	Read 5	346639.4	77.7	15
Read 6	356738.0	49.4	15	Read 6	346636.0	75.3	15

0.50				1.00			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	360629.4	235.4	15	1	360774.0	140.2	15
2	360001.4	122.7	15	2	360424.7	53.7	15
3	359766.0	99.0	15	3	360591.4	58.3	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	360891.4	14622.4	15	0	339124.0	23179.1	15
1	356358.7	11118.4	15	1	349324.7	9070.6	15
3	349575.4	2753.6	15	3	343930.0	2796.3	15
4	353336.0	2006.5	15	4	342601.4	2424.5	15
5	350703.4	1369.2	15	5	337818.0	1676.5	15
6	348484.7	827.7	15	6	332904.7	971.9	15
8	346077.4	435.1	15	7	329133.4	466.5	15
9	344163.4	139.1	15	9	326264.7	266.5	15
10	342587.4	69.1	15	10	323576.0	170.6	15
11	341307.4	94.7	15	11	321818.7	80.5	15
12	340436.7	65.7	15	12	320467.4	85.3	15
14	339538.0	61.4	15	14	319190.0	49.3	15
15	338811.4	85.0	15	15	318194.0	80.2	15
16	338326.7	65.3	15	16	317410.0	53.9	15
17	337721.4	78.2	15	17	316664.7	111.6	15
18	337276.7	110.3	15	18	316086.0	49.6	15
				19	315624.0	72.9	15
Read 1	337168.0	91.1	15	Read 1	315445.4	47.2	15
Read 2	336753.4	119.9	15	Read 2	315335.4	43.5	15
Read 3	336413.4	66.9	15	Read 3	315076.7	55.2	15
Read 4	336161.4	72.7	15	Read 4	314712.7	87.7	15
Read 5	335843.4	69.3	15	Read 5	314624.7	65.3	15
Read 6	335606.0	73.4	15	Read 6	314470.0	84.5	15

5.00				10.00			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	360772.0	114.6	15	1	362862.0	240.1	15
2	360637.4	65.0	15	2	362457.4	108.0	15
3	360774.0	117.2	15	3	362152.0	90.7	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	327764.7	45255.9	15	0	327928.7	34439.0	15
1	315180.7	20716.3	15	1	267945.4	10066.3	15
3	292713.4	6328.1	15	3	249413.3	11219.9	15
4	281653.4	5967.3	15	4	221911.3	9753.4	15
5	261337.3	4349.7	15	5	190505.3	5328.5	15
6	244714.7	2634.8	15	6	169107.3	3216.2	15
7	231539.3	1507.4	15	7	151976.0	1866.9	15
9	221494.7	887.8	15	9	138900.7	1094.5	15
10	214325.3	586.4	15	10	129633.3	727.1	15
11	208680.7	403.4	15	11	122492.0	503.0	15
12	204417.3	279.2	15	12	117100.7	371.2	15
13	201286.0	203.2	15	13	112966.7	283.4	15
15	198946.7	167.6	15	15	109904.7	203.7	15
16	197211.3	105.6	15	16	107391.3	162.1	15
17	195876.7	132.2	15	17	105550.0	119.0	15
18	194973.3	35.0	15	18	104116.0	102.6	15
19	194300.0	45.5	15	19	102920.0	117.5	15
Read 1	193666.0	90.0	15	Read 1	101990.0	133.2	15
Read 2	193286.0	37.2	15	Read 2	101320.7	41.2	15
Read 3	193016.7	53.5	15	Read 3	100816.7	36.3	15
Read 4	192773.3	83.8	15	Read 4	100394.7	36.7	15
Read 5	192578.7	92.9	15	Read 5	100082.0	25.1	15
Read 6	192393.3	67.6	15	Read 6	99843.3	30.0	15

25.00

Blank Reading

Counter	Green Ave	Grn stDev	counts averaged
1	362780.0	159.0	15
2	362402.0	86.6	15
3	362204.0	57.0	15

Analyze Standard...

Mix Counter	Green Ave	Grn stDev	counts averaged
0	312135.4	35826.0	15
1	194530.0	18843.0	15
3	155922.7	15965.0	15
4	111059.3	9449.4	15
5	80874.0	4761.9	15
6	60959.3	2357.8	15
7	47735.3	1257.1	15
9	38716.7	736.5	15
10	32613.3	463.3	15
11	28236.7	310.2	15
12	25118.0	217.5	15
13	22822.7	157.5	15
15	21118.7	116.4	15
16	19829.3	86.6	15
17	18856.7	65.3	15
18	18105.3	48.3	15
19	17532.7	37.7	15
Read 1	17093.3	23.9	15
Read 2	16740.0	17.9	15
Read 3	16454.0	14.5	15
Read 4	16222.0	11.7	15
Read 5	16035.3	8.1	15
Read 6	15908.0	8.3	15

Table C.8. Instrument comparison: Nitrite sensor (5 cm flow-cell)

Sample 1 RAS 4/3 Pre-Backflush				Sample 2 RAS 4/2 45 min (1:100 dilution)			
Blank Reading				Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged	Counter	Green Ave	Grn stDev	counts averaged
1	7513.2	15.4	15	1	8006.0	2.0	2
2	7536.1	7.5	15	2	7997.2	6.2	15
3	7548.0	7.2	11	3	7996.8	29.8	15
Analyze Standard...				Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged	Mix Counter	Green Ave	Grn stDev	counts averaged
0	8094.0	1941.4	15	0	8489.7	1117.4	15
1	7445.7	389.8	15	1	7934.8	501.3	15
3	6714.5	106.0	15	2	7579.6	177.6	15
4	6466.4	33.2	15	3	7560.7	73.9	15
5	6274.8	13.4	15	5	7593.5	37.1	15
6	6110.7	9.2	15	6	7569.1	19.3	15
7	5986.7	10.8	15	7	7527.5	6.1	15
9	5891.5	35.2	15	8	7492.9	6	15
10	5833.6	6.0	15	9	7462	5.9	15
11	5754.7	6.4	15	10	7423.1	6.9	15
12	5760.3	5.9	15	11	7392	7.7	15
13	5735.1	4.1	15	12	7369.2	11.5	15
15	5715.9	4.5	15	13	7349.6	7.8	15
16	5705.1	4.1	15	14	7334.8	8.7	15
17	5697.2	5.3	15	16	7322	8.1	15
18	5687.6	3.5	15	17	7309.9	5	15
19	5680.9	3.4	14	18	7300.3	5.7	15
				19	7288.3	10.1	15
Read 1	5674.8	7.9	15	Read 1	7278.3	4.5	15
Read 2	5671.3	6.1	14	Read 2	7257.9	4.6	15
Read 3	5669.2	4.8	15	Read 3	7254.8	4.9	15
Read 4	5661.2	7.4	15	Read 4	7254	5.7	15
Read 5	5662.8	4.9	15	Read 5	7255.7	5.6	15
Read 6	5652.8	15.4	15	Read 6	7252.4	6.7	15

Sample 3 RAS 4/2 165 min (1:100 dilution)			
Blank Reading			
Counter	Green Ave	Grn stDev	counts averaged
1	7894.8	205.5	15
2	7955.9	8.5	15
3	7944.4	23.1	5
Analyze Standard...			
Mix Counter	Green Ave	Grn stDev	counts averaged
0	8006.5	1835.7	15
1	8804.3	955.5	15
2	7564.3	289.0	15
3	7540.1	127.3	15
5	7624.3	61.5	15
6	7596.7	35.8	15
7	7597.3	6.5	15
8	7568.4	6.9	15
9	7550.1	7.1	15
10	7537.9	8.8	15
11	7526.4	5.3	15
12	7524.4	5.9	15
13	7519.7	5.1	15
14	7512.0	6.4	15
16	7507.9	5.9	15
17	7508.7	4.5	15
18	7508.3	4.0	15
19	7503.6	4.5	15
Read 1	7502.1	3.3	15
Read 2	7497.2	5.9	15
Read 3	7497.9	4.9	15
Read 4	7497.3	3.4	15
Read 5	7500.8	3.6	15
Read 6	7499.7	5.5	15

Table C.9. Nitrite sensor (5 cm flow-cell): Data for global calibration curve

Conc. (μM)	%T	A	RAS samples	%T	A
0	98.59159	0.00616	Pre-Backflush	75.21275	0.12371
0	97.89533	0.00924	45 min (1:100)	90.73563	0.04222
0	97.94289	0.00903	165 min (1:100)	94.32736	0.02536
0	97.22191	0.01224			
0	98.44016	0.00683			
0	97.36631	0.01159			
0	98.10985	0.00829			
0	97.49212	0.01103			
0	98.03611	0.00861			
0	96.59870	0.01503			
0	97.19987	0.01233			
0.1	96.15615	0.01702			
0.1	95.79930	0.01864			
0.1	97.90932	0.00918			
0.1	97.58866	0.01060			
0.1	95.19587	0.02138			
0.1	95.30764	0.02087			
0.1	95.42268	0.02035			
0.2	93.31120	0.03007			
0.2	93.91722	0.02725			
0.2	93.71753	0.02818			
0.2	93.50179	0.02918			
0.2	92.35960	0.03452			
0.3	91.24171	0.03981			
0.3	91.66231	0.03781			
0.3	90.07578	0.04539			
0.3	91.05590	0.04069			
0.3	89.79870	0.04673			
0.5	87.34428	0.05877			
0.5	87.60534	0.05747			
0.5	86.40342	0.06347			
0.5	86.00934	0.06545			
0.5	84.08935	0.07526			
1	72.46048	0.13990			
2	54.92637	0.26022			
2	54.26267	0.26550			

Table C.10. Nitrite sensor (2 cm flow-cell): Data for global calibration curve

Conc. (µM)	A	Conc. (µM)	A	Conc. (µM)	A
0.00	0.00487	0.30	0.02215	5.00	0.27349
0.00	0.00241	0.50	0.02738	5.00	0.27311
0.00	0.00453	0.50	0.03265	5.00	0.27401
0.00	0.00611	0.50	0.03276	5.00	0.27281
0.00	0.00686	0.50	0.03232	10.00	0.53935
0.00	0.00158	0.50	0.02944	10.00	0.52614
0.00	0.00631	0.50	0.02822	10.00	0.54527
0.00	0.00598	0.50	0.02819	10.00	0.54443
0.00	0.00317	0.50	0.02962	10.00	0.55587
0.00	0.00806	0.50	0.03043	10.00	0.54313
0.00	0.00872	0.50	0.02963	10.00	0.54441
0.00	0.00521	0.50	0.03039	10.00	0.54494
0.00	0.01020	0.50	0.03202	10.00	0.53685
0.00	0.00467	1.00	0.05422	15.00	0.77477
0.00	0.00580	1.00	0.05680	15.00	0.87921
0.00	0.00606	1.00	0.06070	15.00	0.81451
0.00	0.00586	1.00	0.05868	25.00	1.31047
0.00	0.00751	1.00	0.05328	25.00	1.32856
0.00	0.00139	1.00	0.05520	25.00	1.30852
0.10	0.00846	1.00	0.05106	25.00	1.31540
0.10	0.00946	1.00	0.05950	25.00	1.32452
0.10	0.01068	1.00	0.05710	25.00	1.27585
0.10	0.01329	1.00	0.05823	25.00	1.27104
0.20	0.01858	1.00	0.05878	25.00	1.38519
0.20	0.01205	1.00	0.05627	25.00	1.38256
0.20	0.01539	1.00	0.05950	25.00	1.34876
0.25	0.01670	1.00	0.05710	25.00	1.34809
0.25	0.01768	1.00	0.05823		
0.25	0.01394	1.00	0.05878		
0.25	0.01454	1.00	0.05627		
0.25	0.01922	1.00	0.05807		
0.25	0.01970	1.00	0.05978		
0.25	0.01605	1.00	0.05958		
0.25	0.01749	1.00	0.06145		
0.25	0.01837	5.00	0.26408		
0.25	0.01789	5.00	0.26118		
0.25	0.01802	5.00	0.27209		
0.30	0.02342	5.00	0.27014		
0.30	0.01909	5.00	0.27170		

2.7. Appendix D: Analytical equations

Equation: Linear calibration curve

$$S = mC + b$$

Where:
S = output signal
C = analyte concentration
m = slope of regression line
b = signal intercept of regression line

Equation: Residual standard deviation (random errors in the *y* direction)

$$s(r) = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n-2}}$$

Equation: Standard deviation of the slope

$$s(m) = \frac{s(r)}{\sqrt{\sum_i (x_i - \bar{x})^2}}$$

Equation: Prediction interval for predicted values

$$s_{x_0} = \frac{s(r)}{m}\sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(\bar{y}_o - \bar{y})^2}{m^2 \sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

Where:
$s(r)$ = residual standard deviation
x_i = value on the *x*-axis (concentration)
$\bar{x}$ = mean of the *x* values
y_i = observed value of *y*
$\hat{y}_i$ = predicted value of *y*
$\bar{y}_o$ = mean signal value of N replicate measurements
n = number of calibration points
N = number of replicate measurements
m = slope

Equation: Sensitivity as detection limit

$$S = \frac{3s_x}{m}$$

Equation: Recovery

$$\%R = \frac{F - I}{I} \times 100$$

Where:
F = method blank signal
I = method blank signal after spiked sample run